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(54) **Methods of producing secreted receptor analogs and biologically active peptide dimers.**

(57) Methods for producing a secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof. Pharmaceutical and diagnostic compositions utilizing the polypeptides are also disclosed.

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METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE PEPTIDE DIMERS

Technical Field

The present invention is generally directed toward the expression of proteins, and more specifically, toward the expression of growth factor receptor analogs and biologically active peptide dimers.

Background of the Invention

In higher eucaryotic cells, the interaction between receptors and ligands (e.g., hormones) is of central importance in the transmission of and response to a variety of extracellular signals. It is generally accepted that hormones and growth factors elicit their biological functions by binding to specific recognition sites (receptors) in the plasma membranes of their target cells. Upon ligand binding, the receptor undergoes a conformational change, triggering secondary cellular responses that result in the activation or inhibition of intracellular processes. The stimulation or blockade of such an interaction by pharmacological means has important therapeutic implications for a wide variety of illnesses.

Ligands fall into two classes: those that have stimulatory activity, termed agonists; and those that block the effects elicited by the original ligands, termed antagonists. The discovery of agonists that differ in structure and composition from the original ligand may be medically useful. In particular, agonists that are smaller than the original ligand may be especially useful. The bioavailability of these smaller agonists may be greater than that of the original ligand. This may be of particular importance for topical applications and for instances when diffusion of the agonist to its target sites is inhibited by poor circulation. Agonists may also have slightly different spectra of biological activity and/or different potencies, allowing them to be used in very specific situations. Agonists that are smaller and chemically simpler than the native ligand may be produced in greater quantity and at lower cost. The identification of antagonists which specifically block, for example, growth factor receptors has important pharmaceutical applications. Antagonists that block receptors against the action of endogenous, native ligand may be used as therapeutic agents for conditions including atherosclerosis, autocrine tumors, fibroplasia and keloid formation.

The discovery of new ligands that may be used in pharmaceutical applications has centered around designing compounds by chemical modification, complete synthesis, and screening potential ligands

by complex and costly screening procedures. The process of designing a new ligand usually begins with the alteration of the structure of the original effector molecule. If the original effector molecule is known to be chemically simple, for example, a catecholamine or prostaglandin, the task is relatively straightforward. However, if the ligand is structurally complex, for example, a peptide hormone or a growth factor, finding a molecule which is functionally equivalent to the original ligand becomes extremely difficult.

Currently, potential ligands are screened using radioligand binding methods (Lefkowitz et al., *Biochem. Biophys. Res. Comm.* 60: 703-709, 1974; Aurbach et al., *Science* 188: 1223-1225, 1974; Atlas et al., *Proc. Natl. Acad. Sci. USA* 71: 4246-4248, 1974). Potential ligands can be directly assayed by binding the radiolabeled compounds to responsive cells, to the membrane fractions of disrupted cells, or to solubilized receptors. Alternatively, potential ligands may be screened by their ability to compete with a known labeled ligand for cell surface receptors.

The success of these procedures depends on the availability of reproducibly high quality preparations of membrane fractions or receptor molecules, as well as the isolation of responsive cell lines. The preparation of membrane fractions and soluble receptor molecules involves extensive manipulations and complex purification steps. The isolation of membrane fractions requires gentle manipulation of the preparation, a procedure which does not lend itself to commercial production. It is very difficult to maintain high biological activity and biochemical purity of receptors when they are purified by classical protein chemistry methods. Receptors, being integral membrane proteins, require cumbersome purification procedures, which include the use of detergents and other solvents that interfere with their biological activity. The use of these membrane preparations in ligand binding assays typically results in low reproducibility due to the variability of the membrane preparations.

As noted above, ligand binding assays require the isolation of responsive cell lines. Often, only a limited subset of cells is responsive to a particular agent, and such cells may be responsive only under certain conditions. In addition, these cells may be difficult to grow in culture or may possess a low number of receptors. Currently available cell types responsive to platelet-derived growth factor (PDGF), for example, contain only a low number (up to 4×10^5 ; see Bowen-Pope and Ross, *J. Biol. Chem.* 257: 5161-5171, 1982) of receptors per cell,

thus requiring large numbers of cells to assay PDGF analogs or antagonists.

Presently, only a few naturally-occurring secreted receptors, for example, the interleukin-2 receptor (IL-2-R) have been identified. Rubin et al. (J. Immun. 135: 3172-3177, 1985) have reported the release of large quantities of IL-2-R into the culture medium of activated T-cell lines. Bailon et al. (Bio/Technology 5: 1195-1198, 1987) have reported the use of a matrix-bound interleukin-2 receptor (IL-2-R) to purify recombinant interleukin-2.

Three other receptors have been secreted from mammalian cells. The insulin receptor (Ellis et al., J. Cell Biol. 150: 14a, 1987), the HIV-1 envelope glycoprotein cellular receptor CD4 (Smith et al., Science 238: 1704-1707, 1987) and the epidermal growth factor (EGF) receptor (Livneh et al., J. Biol. Chem. 261: 12490-12497, 1986) have been secreted from mammalian cells using truncated cDNAs that encode portions of the extracellular domains.

There is therefore a need in the art for a method of producing secreted receptors. There is a further need in the art for an assay system that permits high volume screening of compounds that may act on higher eucaryotic cells via specific surface receptors. This assay system should be rapid, inexpensive and adaptable to high volume screening. The present invention discloses such a method and assay system, and further provides other related advantages.

Disclosure of Invention

Briefly stated, the present invention discloses methods for producing secreted receptor analogs including ligand-binding receptor analogs and secreted platelet-derived growth factor receptor (PDGF-R) analogs. In addition, the present invention discloses methods for producing secreted peptide dimers.

Within one aspect of the invention a method for producing a secreted PDGF-R analog is disclosed, comprising (a) introducing into a host cell a DNA construct capable of directing the expression and secretion of a PDGF receptor analog, the DNA construct containing a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream in proper reading frame by a DNA sequence encoding at least a portion of the extracellular domain of a PDGF-R, the portion including a ligand-binding domain; (b) growing the host cell in an appropriate growth medium; and (c) isolating the PDGF-R analogs from the host cell.

Within one embodiment of the present invention, a PDGF-R analog comprising the amino acid

sequence of Figure 1 from isoleucine, number 29 to methionine, number 441 is secreted. Within another embodiment a PDGF-R analog comprising the amino acid sequence of Figure 1 from isoleucine, number 29 to lysine, number 531 is secreted.

Yet another aspect of the present invention discloses a method for producing a secreted, biologically active peptide dimer. The method generally comprises a) introducing into a host cell a DNA construct capable of directing the expression and secretion of a peptide requiring dimerization for biological activity, the DNA construct containing a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of the peptide; and (c) isolating the biologically active peptide dimer from the host cell.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active peptide dimer, comprising (a) introducing into a host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain, selected from the group consisting of C_{H1} , C_{H2} , C_{H3} , $C_{\gamma1}$, $C_{\gamma2}$, $C_{\gamma3}$, $C_{\gamma4}$, and μ , joined to an immunoglobulin heavy chain hinge region; (c) growing the host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of the biologically active peptide dimer; and (d) isolating the biologically active peptide dimer from the host cell.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active peptide dimer, comprising (a) introducing into a host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to at least one immunoglobulin heavy chain constant region domain, selected from the group consisting of C_{H1} , C_{H2} , C_{H3} , $C_{\gamma1}$, $C_{\gamma2}$, $C_{\gamma3}$, $C_{\gamma4}$, and μ , joined to an

immunoglobulin heavy chain hinge region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of the biologically active peptide dimer; and (d) isolating the biologically active peptide dimer from the host cell.

In yet another aspect of the invention, a method is disclosed for producing a secreted, ligand-binding receptor analog, comprising (a) introducing into a host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding receptor analog joined to at least an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ, joined to an immunoglobulin heavy chain hinge region; (c) growing the host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of the ligand-binding receptor analog; and (d) isolating the ligand-binding receptor analog from the host cell.

In another aspect of the invention, a method is disclosed for producing a secreted, ligand-binding receptor analog, comprising (a) introducing into a host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream in proper reading frame by a DNA sequence encoding a ligand-binding receptor analog joined to at least one immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ, joined to an immunoglobulin heavy chain hinge region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of the ligand-binding receptor analog; and (d) isolating the ligand-binding receptor analog from the host cell.

Methods disclosed in the present invention may include, after the step of isolating the receptor analogs and biologically active peptide dimers, purifying the analogs and dimers. Purification methods include gel filtration, ion exchange chromatography, and immunoaffinity chromatography.

Host cells for use in the present invention include cultured mammalian cells and fungal cells. In a preferred embodiment strains of the yeast *Saccharomyces cerevisiae* are used as host cells. Within another preferred embodiment cultured mouse myeloma cells are used as host cells.

In one embodiment the ligand-binding receptor analog consists essentially of the PDGF-R extracellular domain. PDGF-R analogs produced by the above-disclosed methods may be used, for instance, within a method for determining the presence of human PDGF or an isoform thereof in a biological sample, or within a method for purifying human PDGF or an isoform thereof from a sample.

A method for determining the presence of human PDGF or an isoform thereof in a biological sample is disclosed and comprises (a) incubating a polypeptide comprising a human PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of containing human PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and (b) detecting the presence of receptor/ligand complexes, and therefrom determining the presence of human PDGF or an isoform thereof. Suitable biological samples in this regard include blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media, and chemically or physically separated portions thereof.

A method is disclosed for purifying human PDGF or an isoform thereof from a sample, which comprises (a) immobilizing a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein on a substrate; (b) contacting the sample containing human PDGF or an isoform thereof with the immobilized polypeptide under suitable conditions such that the human PDGF or isoform thereof binds to the polypeptide; and (c) eluting the human PDGF or isoform thereof from the polypeptide. Suitable samples include the biological samples discussed above.

The present invention also discloses pharmaceutical compositions comprising a human PDGF receptor analog fused to a dimerizing protein in combination with a physiologically carrier or diluent.

Within a related aspect of the present invention, a diagnostic composition, comprising a PDGF-R analog fused to a dimerizing protein tagged with a label capable of providing a detectable signal, is disclosed. Suitable labels in this regard include

iodine-125 or technetium-99.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawing

Figure 1 illustrates the nucleotide sequence of the PDGF receptor cDNA and the derived amino acid sequence of the primary translation product. Numbers above the lines refer to the nucleotide sequence; numbers below the lines refer to the amino acid sequence.

Figure 2 illustrates the construction of pBTL10, pBTL11 and pBTL12.

Figure 3 illustrates the construction of pCBS22.

Figure 4 illustrates the construction of pBTL13 and pBTL14.

Figure 5 illustrates the construction of pBTL15.

Figure 6 illustrates the construction of pBTL22 and pBTL26.

Figure 7 illustrates the construction of pSDL114. Symbols used are S.S., signal sequence; C_k, immunoglobulin light chain constant region sequence; μ prom, μ promote, μ enh; μ enhancer.

Figure 8 illustrates the construction of pSDB113. Symbols used are S.S., signal sequence; C_{H1}, C_{H2}, C_{H3}, immunoglobulin heavy chain constant region domain sequences; H, immunoglobulin heavy chain hinge region; M, immunoglobulin membrane anchor sequences; C_{Y1M}, immunoglobulin heavy chain constant region and membrane anchor sequences.

Figure 9 illustrates the construction of pBTL15, pBTL14, Pgl-Neo, pIC05V_kHuC_k-neo. Symbols used are set forth in figures 7 and 8, and also include L_H, mouse immunoglobulin heavy chain signal sequence; V_H, mouse immunoglobulin heavy chain variable region sequence; E, mouse immunoglobulin heavy chain enhancer; L_k, mouse immunoglobulin light chain signal sequence; 05V_k, mouse immunoglobulin light chain variable region sequence; Neo^R, neomycin resistance gene.

Best Mode for Carrying Out the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded that has been modified through human intervention to contain segments of DNA combined and jux-

taposed in a manner that as a whole would not otherwise exist in nature.

Secretory Signal Sequence: A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast Barrier protein.

Platelet-Derived Growth Factor Receptor (PDGF-R) Analog: A portion of a PDGF receptor capable of binding anti-PDGF receptor antibodies, PDGF, PDGF isoforms, PDGF analogs, or PDGF antagonists.

Dimerizing Protein: A polypeptide chain having affinity for a second polypeptide chain, such that the two chains associate to form a dimer that has an additional activity independent of either of the polypeptide chains as monomers. The second polypeptide chain may be the same or a different chain.

Biological activity: A function or set of activities performed by a molecule in a biological context (i.e., in an organism or an *in vitro* facsimile). Biological activities may include the induction of extracellular matrix secretion from responsive cell lines, the induction of hormone secretion, the induction of chemotaxis, the induction of mitogenesis, the induction of differentiation, or the inhibition of cell division of responsive cells.

Ligand: A molecule, other than an antibody or an immunoglobulin, capable of being bound by the ligand-binding domain of a receptor. The molecule may be chemically synthesized or may occur in nature.

Joined: Two or more DNA coding sequences are said to be joined when, as a result of in-frame fusions between the DNA coding sequences or as a result of the removal of intervening sequences by normal cellular processing, the DNA coding sequences are translated into a polypeptide fusion.

As noted above, the present invention provides methods for producing secreted receptor analogs including ligand-binding receptor analogs and PDGF receptor analogs. Secreted receptor analogs may be used to screen for new compounds that act as agonists or antagonists when interacting with cells containing membrane-bound receptors. In addition, the methods of the present invention provide peptides of therapeutic value that are biologically active only as dimers. Moreover, the present inven-

tion provides methods of producing peptide dimers that are biologically active only as non-covalently associated dimers. Secreted, biologically active dimers that may be produced using the present invention include nerve growth factor, colony stimulating factor-1, factor XIII, and transforming growth factor β .

Ligand-binding receptor analogs that may be used in the present invention include the ligand-binding domains of the epidermal growth factor receptor (EGF-R) and the insulin receptor. As used herein, a ligand-binding domain is that portion of the receptor that is involved in binding ligand and is generally a portion or essentially all of the extracellular domain that extends from the plasma membrane into the extracellular space. The ligand-binding domain of the EGF-R, for example, resides in the extracellular domain. EGF-R dimers have been found to exhibit higher ligand-binding affinity than EGF-R monomers (Boni-Schnetzler and Pilch, *Proc. Natl. Acad. Sci. USA* 84:7832-7836, 1987). The insulin receptor (Ullrich et al., *Nature* 313:758-761, 1985) requires dimerization for biological activity.

Another example of a receptor that may be secreted from a host cell is a platelet-derived growth factor receptor (PDGF-R). A complementary DNA that encodes a PDGF-R with a primary translation product of 190 KDa has been cloned (Gronwald et al., *Proc. Natl. Acad. Sci. USA* 85:3435-3439, 1988). The receptor includes an extracellular domain implicated in the ligand-binding process, a transmembrane domain, and a cytoplasmic domain containing a tyrosine kinase activity. PDGF-R is capable of binding any or all combinations of native PDGF or its isoforms. (PDGF is a disulfide-bonded, two-chain molecule, which is made up of an A chain and a B chain. These chains may be combined as AB heterodimers, AA homodimers or BB homodimers. These dimeric molecules are referred to herein as "isoforms".)

A secreted PDGF-R (sPDGF-R) can be readily employed in studies to characterize the PDGF-R. PDGF-R may be characterized, for example, by identifying ligands other than PDGF and its isoforms, by competition assays using different ligands and by modifying the sPDGF-R to define domains of the receptor that are critical for ligand binding. These studies are necessary and lead to the systematic designing of novel, PDGF-like agonists and antagonists. The sPDGF-R also offers a source of large amounts of the receptor protein for use in ligand screening procedures. The sPDGF-R may also be used in radioligand binding assays to compete with samples containing PDGF. The use of sPDGF-R as a therapeutic agent has the advantage of high receptor affinity and specificity for PDGF. As an antagonist, the sPDGF-R is a poten-

tial drug for atherosclerosis. PDGF, which is implicated in the pathogenesis of atherosclerotic plaques, may be blocked by the therapeutic use of sPDGF-R, thus preventing plaque formation. sPDGF-R may also be employed to produce a battery of novel antibodies that may be used both *in vivo* and *in vitro*. Examples of *in vivo* use of PDGF-R antibodies include the use of PDGF-R blocking antibodies in atherosclerosis therapy or the use of antibodies that have an agonist character in wound healing. *In vitro*, sPDGF-R antibodies may be employed in a variety of tests and assay procedures, for example, Western blots, ELISA assays and immunopurification. The sPDGF-R molecules may also be applied in the purification of PDGF by taking advantage of the ligand-receptor affinity interaction.

The present invention also provides a standardized assay system for determining the presence of PDGF, PDGF isoforms, PDGF agonists or PDGF antagonists not previously available in the art. This assay system provides secreted PDGF receptor analogs that may be utilized in a variety of screening assays.

As noted above, the present invention provides methods for producing peptide dimers that require dimerization for biological activity or enhancement of biological activity. Peptides requiring dimerization for biological activity include nerve growth factor, colony-stimulating factor-1 (CSF-1), transforming growth factor β (TGF- β), PDGF, and factor XIII. Nerve growth factor is a non-covalently linked dimer (Harper et al., *J. Biol. Chem.* 257:8541-8548, 1982). CSF-1, which specifically stimulates the proliferation and differentiation of cells of mononuclear phagocytic lineage, is a disulfide-bonded homodimer (Rettermier et al., *Mol. Cell. Biol.* 7:2378-2387, 1987). TGF- β is biologically active as a disulfide-bonded dimer (Assoian et al., *J. Biol. Chem.* 258:7155-7160, 1983). Factor XIII is a plasma protein that exists as a two chain homodimer in its activated form (Ichinose et al., *Biochem.* 25:6900-6906, 1986). PDGF, as noted above, is a disulfide-bonded, two chain molecule (Murray et al., U.S. Patent 4,766,073).

The present invention provides methods by which receptor analogs including ligand-binding receptor analogs and PDGF-R analogs requiring dimerization for activity may be secreted from host cells. The methods described herein are particularly advantageous in that they allow the production of large quantities of purified receptors. The receptors may be used in assays for the screening of potential ligands, in assays for binding studies, as imaging agents, and as agonists and antagonists within therapeutic agents.

A DNA sequence encoding a human PDGF receptor may be isolated as a cDNA using tech-

niques known in the art (see, for example, Okayama and Berg, Mol. Cell. Biol. 2 : 161-170, 1982; Mol. Cell. Biol. 3: 280-289, 1983). A cDNA encoding a PDGF-R has been cloned from a diploid human dermal fibroblast cDNA library using oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Gronwald et al., *ibid.*). In a preferred embodiment, a DNA sequence encoding a PDGF receptor analog consisting essentially of the extracellular domain of a PDGF receptor is used, although smaller DNA sequences encoding portions of at least 400 amino acids of the extracellular domain may be used.

DNA sequences encoding EGF-R (Ullrich et al., Nature 304:418-425, 1984), the insulin receptor (Ullrich et al., Nature 313:756-761, 1985), nerve growth factor (Ullrich et al., Nature 303:821-825, 1983), colony stimulating factor-1 (Rottenmiller et al., *ibid.*), transforming growth factor β (Derynck et al., Nature 316:701-705, 1985), PDGF (Murray et al., *ibid.*), and factor XIII (Ichinose et al., *ibid.*) may also be used within the present invention.

To direct peptides requiring dimerization for biological activity or receptor analogs into the secretory pathway of the host cell, at least one secretory signal sequence is used in conjunction with the DNA sequence of interest. Preferred secretory signals include the alpha factor signal sequence (pre-pro sequence) (Kurjan and Herkowitz, Cell 30: 933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake EP 116,201, 1983), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670) and the mouse immunoglobulin V_H signal sequence (Loh et al., Cell 33:85-93, 1983). Particularly preferred signal sequences are the SUC2 signal sequence (Carlson et al., Mol. Cell. Biol. 3:439-447, 1983) and the PDGF receptor signal sequence. The signal sequence may be used singly or combined with a sequence encoding the third domain of Barrier (described in co-pending commonly assigned U.S. Patent Application Serial No. 104,316, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the DNA sequence and in proper reading frame with both the secretory signal sequence and the DNA sequence of interest.

In one embodiment of the present invention, a sequence encoding a dimerizing protein is joined to a sequence encoding a peptide dimer or a receptor analog. As shown herein, the present invention utilizes such an arrangement to drive the association of the peptide or receptor to form a biologically active molecule upon secretion. Suitable dimerizing proteins include the *S. cerevisiae*

repressible acid phosphatase (Mizunaga et al., J. Biochem. (Tokyo) 103:321-326, 1988), the *S. cerevisiae* type 1 killer preprotoxin (Sturley et al., EMBO J. 5:3381-3390, 1986), the *S. caltsbergensis* alpha galactosidase melibiase (Sumner-Smith et al., Gene 36:333-340, 1985), and the *Neurospora crassa* ornithine decarboxylase (Digangi et al., J. Biol. Chem. 262:7889-7893, 1987). In this regard, sequences encoding an immunoglobulin heavy chain hinge region (Takahashi et al., Cell 29:671-679, 1982), the *S. cerevisiae* SUC2 gene (Carlson et al., Mol. Cell. Biol. 3:439-447, 1983), immunoglobulin gene sequences, and portions thereof are preferred. In one embodiment of the invention, immunoglobulin gene sequences are used to drive the association of non-immunoglobulin peptides. Immunoglobulin constant regions comprise discrete domains that show similarity in their three dimensional conformation. These discrete domains correspond to the immunoglobulin heavy chain constant region domain exons in the immunoglobulin gene (Hood et al., in Immunology, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA; Honjo et al., Cell 18:559-568, 1979; and Takahashi et al., Cell 29:671-679, 1982). In this regard, it is particularly preferred to use an immunoglobulin light chain constant region in combination with at least one immunoglobulin heavy chain constant region domain joined to an immunoglobulin hinge region as the dimerizing protein. Immunoglobulin heavy chain constant region domains include C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ . A particularly preferred immunoglobulin heavy chain constant region domain is C_H1.

Host cells for use in practicing the present invention include mammalian and fungal cells. Fungal cells, including species of yeast (e.g., *Saccharomyces* spp., *Schizosaccharomyces* spp.), or filamentous fungi (e.g., *Aspergillus* spp., *Neurospora* spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76: 1035-1039, 1978), YEpl3 (Broach et al., Gene 8: 121-133 1979), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al. *ibid.*), URA3 (Botstein et al., Gene 8: 17, 1979), HIS3 (Struhl et al., *ibid.*)

or POT1 (Kawasaki and Bell, EP 171,142). Other suitable selectable markers include the CAT gene, which confers chloramphenicol resistance on yeast cells, or the lacZ gene, which results in blue colonies when active β -galactosidase is expressed.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255: 12073-12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet.* 1: 419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol.* 101: 192-201, 1983). In this regard, particularly preferred promoters are the TPI1 promoter (Kawasaki, U.S. Patent No. 4,599,311, 1988) and the ADH2-4^s promoter (Russell et al., *Nature* 304, 652-654, 1983 and Irani and Kilgore, described in pending, commonly assigned U.S. Patent Application Serial Nos. 029,867 and 183,130, which are incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, *ibid.*).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi *Aspergillus* (McKnight and Upshall, described in pending, commonly assigned U.S. Patent Application Serial Nos. 820,519 and 942,494 corresponding to published European Patent Application EP272,277, which are incorporated herein by reference). Examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the ADH3 promoter (McKnight et al., *EMBO J.* 4: 2093-2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., *ibid.*). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of *Aspergillus*.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (*ibid.*), Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75: 1929-1933, 1978), Yelton et al., (*Proc. Natl. Acad. Sci. USA* 81:1740-1747, 1984), and Russell (*Nature* 301: 167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In a preferred embodiment, a yeast host cell that contains a genetic deficiency in a gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, the yeast host

cell contains a genetic deficiency in the MNN9 gene (described in pending, commonly assigned U.S. Patent Application No. 116,095, which is incorporated by reference herein in its entirety). Most preferably, the yeast host cell contains a disruption of the MNN9 gene. Yeast host cells having such defects may be prepared using standard techniques of mutation and selection. Ballou et al. (*J. Biol. Chem.* 255: 5986-5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized yeast cells were screened using fluoresceinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties. To optimize production of the heterologous proteins, it is preferred that the host strain carries a mutation, such as the yeast pep4 mutation (Jones, *Genetics* 85: 23-33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred mammalian cell lines include the COS-1 (ATCC CRL 1650), BHK, p363.Ag.8.653 (ATCC CRL 1580), FO (ATCC CRL 1846) and 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, 1977) cell lines. A preferred BHK cell line is the tk⁻ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79:1106-1110, 1982). A particularly preferred cell line is the SP2/0-Ag14 (ATCC CRL 1581). In addition, a number of other cell lines may be used within the present invention, including Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CC 29.1) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Preferred viral promoters include the major late promoter from adenovirus 2 (Kaufman and Sharp, *Mol. Cell. Biol.* 2:1304-1319, 1982) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1:854-864, 1981). Preferred cellular promoters include the mouse metallothionein I promoter (Palmiter et al., *Science* 222:809-814, 1983) and the mouse V_k promoter (Grant et al., *Nuc. Acids Res.* 15:5498, 1987). A particularly preferred promoter is the mouse V_H promoter (Loh et al., *ibid.*). Such expression vectors may also contain a set of RNA splice sites

located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the insertion site. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9:3719-3730, 1981). A particularly preferred polyadenylation signal is the V_H gene terminator (Loh et al., *ibid.*). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer. Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may then be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wiger et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., EMBO J. 1:841-845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a gene that confers a selectable phenotype (a selectable marker) is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. A particularly preferred amplifiable marker is the DHFR^r cDNA (Simonsen and Levinson, Proc. Natl. Acad. Sci. USA 80:2495-2499, 1983). Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA) and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent 4,713,339). It may also be ad-

vantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Host cells containing DNA constructs of the present invention are grown in an appropriate growth medium. The growth medium is generally a medium that selects for cells containing the DNA construct. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. Yeast cells, for example, are preferably grown in a chemically defined medium, comprising a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 6.5. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. A preferred buffering agent is succinic acid. Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M., preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The culture medium from appropriately grown transformed or transfected host cells is separated from the cell material, and the presence of peptide dimers or secreted receptor analogs is demonstrated. A preferable method of detecting PDGF receptor analogs, for example, is by the binding of the receptors or portions of receptors to a receptor-specific antibody. A particularly preferred antibody is PR7212, a mouse anti-PDGF receptor monoclonal antibody. Another particularly preferred antibody, which may be used for detecting substance P tagged peptides and proteins, is a commercially available rat anti-substance P monoclonal antibody which may be utilized to visualize pep-

tides or proteins that are fused to the C-terminal amino acids of substance P. Ligand binding assays may also be used to detect the presence of receptor analogs. In the case of PDGF receptor analogs, it is preferable to use fetal foreskin fibroblasts, which express PDGF receptors, to compete against the PDGF receptor analogs of the present invention for labelled PDGF and PDGF isoforms.

Assays for detection of secreted, biologically active peptide dimers and receptor analogs may include Western transfer, protein blot or colony filter. A Western transfer filter may be prepared using the method essentially described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350-4354, 1979). Briefly, samples are electrophoresed in a sodium dodecylsulfate polyacrylamide gel. The proteins are electrophoretically transferred to nitrocellulose paper. Protein blot filters may be prepared by filtering supernatant samples or concentrates through nitrocellulose filters using, for example, a Minifold (Schleicher & Schuell). Colony filters may be prepared by growing colonies on a nitrocellulose filter that has been laid across an appropriate growth medium. In this method, a solid medium is preferred. The cells are allowed to grow on the filters for at least 12 hours. The cells are removed from the filters by washing with an appropriate buffer that does not remove the proteins bound to the filters. A preferred buffer comprises 25 mM Tris-base, 19 mM glycine, pH 8.3, 20% methanol.

The peptide dimers and receptor analogs present on the Western transfer, protein blot or colony filters may be visualized by specific antibody binding using methods known in the art. For example, Towbin et al. (*ibid.*) describe the visualization of proteins immobilized on nitrocellulose filters with a specific antibody followed by a labeled second antibody, directed against the first antibody. Kits and reagents required for visualization are commercially available, for example, from Vector Laboratories, (Burlingame, CA), and Sigma Chemical Company (St. Louis, MO).

Secreted, biologically active peptide dimers and receptor analogs may be isolated from the medium of host cells grown under conditions that allow the secretion of the receptor analogs and biologically active peptide dimers. The cell material is removed from the culture medium, and the biologically active peptide dimers and receptor analogs are isolated using isolation techniques known in the art. Suitable isolation techniques include precipitation and fractionation by a variety of chromatographic methods, including gel filtration, ion exchange chromatography and immunoaffinity chromatography. A particularly preferred purification method is immunoaffinity chromatography using an antibody directed against the receptor ana-

log or peptide dimer. The antibody is preferably immobilized or attached to a solid support or substrate. A particularly preferred substrate is CNBr-activated Sepharose (Pharmacia, Piscataway, NJ). By this method, the medium is combined with the antibody/substrate under conditions that will allow binding to occur. The complex may be washed to remove unbound material, and the receptor analog or peptide dimer is released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods of elution include changes in pH, wherein the immobilized antibody has a high affinity for the ligand at a first pH and a reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs of the present invention can be used within a variety of assays for detecting the presence of native PDGF, PDGF isoforms or PDGF-like molecules. These assays will typically involve combining PDGF receptor analogs, which may be bound to a solid substrate such as polymeric microtiter plate wells, with a biological sample under conditions that permit the formation of receptor/ligand complexes. Detection may be achieved through the use of a label attached to the receptor or through the use of a labeled antibody which is reactive with the receptor. Alternatively, the labeled antibody may be reactive with the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Complexes may also be detected visually, i.e., in immunoprecipitation assays, which do not require the use of a label.

Secreted PDGF receptor analogs of the present invention may also be labeled with a radioisotope or other imaging agent and used for *in vivo* diagnostic purposes. Preferred radioisotope imaging agents include iodine-125 and technetium-99, with technetium-99 being particularly preferred. Methods for producing protein-isotope conjugates are well known in the art, and are described by, for example, Eckelman et al. (U.S. Patent No. 4,652,440), Parker et al. (WO 87/05030) and Wilber et al. (EP 203,784). Alternatively, the secreted receptor analogs may be bound to spin label enhancers and used for magnetic resonance (MR) imaging. Suitable spin label enhancers include stable, sterically hindered, free radical compounds such as nitroxides. Methods for labeling ligands for MR imaging are disclosed by, for example, Coffman et al. (U.S. Patent No. 4,656,028). For administration, the labeled receptor analogs are combined with a pharmaceutically acceptable carrier or diluent, such as sterile saline or sterile water. Administration is preferably by bolus injection, preferably intravenously. These imaging agents are particularly useful in identifying the locations of atherosclerotic

plaques, PDGF-producing tumors, and receptor-bound PDGF.

The secreted PDGF receptor analogs of the present invention may also be utilized within diagnostic kits. Briefly, the subject receptor analogs are preferably provided in a lyophilized form or immobilized onto the walls of a suitable container, either alone or in conjunction with additional antibodies capable of binding to native PDGF or selected PDGF isoform(s) or specific ligands. The antibodies, which may be conjugated to a label or unconjugated, are generally included in the kits with suitable buffers, such as phosphate, stabilizers, inert proteins or the like. Generally, these materials are present in less than about 5% weight based upon the amount of active receptor analog, and are usually present in an amount of at least about 0.001% weight. It may also be desirable to include an inert excipient to dilute the active ingredients. Such an excipient may be present from about 1% to 99% weight of the total composition. In addition, the kits will typically include other standard reagents, instructions and, depending upon the nature of the label involved, reactants that are required to produce a detectable product. Where an antibody capable of binding to the receptor or receptor/ligand complex is employed, this antibody will usually be provided in a separate vial. The antibody is typically conjugated to a label and formulated in an analogous manner with the formulations briefly described above. The diagnostic kits, including the containers, may be produced and packaged using conventional kit manufacturing procedures.

As noted above, the secreted PDGF receptor analogs of the present invention may be utilized within methods for purifying PDGF from a variety of samples. Within a preferred method, the secreted PDGF receptor analogs are immobilized or attached to a substrate or support material, such as polymeric tubes, beads, polysaccharide particulates, polysaccharide-containing materials, polyacrylamide or other water insoluble polymeric materials. Methods for immobilization are well known in the art (Mosbach et al., U.S. Patent No. 4,415,865; Clarke et al., *Meth. Enzymology* 88: 438-442, 1979). A common method of immobilization is CNBr activation. Activated substrates are commercially available from a number of suppliers, including Pharmacia (Piscataway, NJ), Pierce Chemical Co. (Rockford, IL) and Bio-Rad Laboratories (Richmond, CA). A preferred substrate is CNBr-activated Sepharose (Pharmacia, Piscataway, NJ). Generally, the substrate/receptor analog complex will be in the form of a column. The sample is then combined with the immobilized receptor analog under conditions that allow binding to occur. The substrate with immobilized receptor analog is

first equilibrated with a buffer solution of a composition in which the receptor analog has been previously found to bind its ligand. The sample, in the solution used for equilibration, is then applied to the substrate/receptor analog complex. Where the complex is in the form of a column, it is preferred that the sample be passed over the column two or more times to permit full binding of ligand to receptor analog. The complex is then washed with the same solution to elute unbound material. In addition, a second wash solution may be used to minimize nonspecific binding. The bound material may then be released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods include changes in pH, wherein the immobilized receptor has a high affinity for PDGF at a first pH and reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs fused to dimerizing proteins of the present invention may be used in pharmaceutical compositions for topical or intravenous application. The PDGF receptor analogs fused to dimerizing proteins are used in combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include saline and sterile water. Pharmaceutical compositions may also contain stabilizers and adjuvants. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Enzymes, including restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase, were obtained from New England Biolabs (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD) and Boehringer-Mannheim Biochemicals (Indianapolis, IN) and were used as directed by the manufacturer or as described in Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY, 1982).

Example 1

Cloning of the PDGF Receptor cDNA

Complementary DNA (cDNA) libraries were prepared from poly(A) RNA from diploid human dermal fibroblast cells, prepared by explant from a normal adult, essentially as described by Hagen et al. (Proc. Natl. Acad. Sci. USA 83: 2412-2416, 1986). Briefly, the poly(A) RNA was primed with oligo(T) and was cloned into λ gt11 using Eco RI linkers. The random primed library was screened for the presence of human PDGF receptor cDNA's using three oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Yarden et al., Nature 323: 226-232, 1986). Approximately one million phage from the random primed human fibroblast cell library were screened using oligonucleotides ZC904, ZC905 and ZC906 (Table 1). Eight positive clones were identified and were plaque purified. Two clones, designated RP41 and RP51, were selected for further analysis by restriction enzyme mapping and DNA sequence analysis. RP51 was found to contain 356 bp of 5'-noncoding sequence, the ATG translation initiation codon and 738 bp of the amino terminal coding sequence. RP41 was found to overlap clone RP51 and contained 2649 bp encoding amino acids 43-925 of the receptor protein.

Table 1

Oligonucleotides Sequences

ZC582 5' AAT TCC CGG G 3'
 ZC583 5' GAT CCC CGG G 3'
 ZC904 5' CAT GGG CAC GTA ATC TAT AGA
 TTC ATC CTT GCT CAT ATC CAT GTA 3'
 ZC905 5' TCT TGC CAG GGC ACC TGG GAC
 ATC TGT TCC CAC ATG ACC GG 3'
 ZC906 5' AAG CTG TCC TCT GCT TCA GCC
 AGA GGT CCT GGG CAG CC 3'
 ZC1380 5' CAT GGT GGA ATT CCT GCT GAT
 3'
 ZC1453 5' AAT TCA TTA TGT TGT TGC AAG
 CCT TCT TGT TCC TGC TAG CTG GTT TCG
 CTG TTA A 3'
 ZC1454 5' GAT CTT AAC AGC GAA ACC AGC
 TAG CAG GAA CAA GAA GGC TTG CAA CAA
 CAT AAT G 3'
 ZC1478 5' ATC GCG AGC ATG CAG ATC
 TGA 3'
 ZC1479 5' AGC TTC AGA TCT GCA TGC TGC
 CGA T
 ZC1776 5' AGC TGA GCG CAA ATG TTG TGT
 CGA GTG CCC ACC GTG CCC AGC TTA GAA

TTC T 3'

ZC1777 5' CTA GAG AAT TGT AAG CTG
 GGC AAC GTG GGC ACT CGA CAC AAC ATT
 TGC GCT G 3'
 5 ZC1846 5' GAT CGG CCA CTG TCG GTG
 CGC TGC ACG CTG CGC AAC GCT GTG GGC
 CAG GAC ACG CAG GAG GTC ATC GTG GTG
 CCA CAC TCC TTG CCC TTT AAG CA 3'
 ZC1847 5' AGC TTG CTT AAA GGG CAA
 10 GGA GTG TGG CAC CAC GAT GAC CTC CTG
 CGT GTC CTG GCC CAC AGC GTT GCG CAG
 CGT GCA GCG CAC CGA CAG TGG CC 3'
 ZC1888 5' CCA GTG CCA AGC TTG TCT AGA
 CTT ACC TTT AAA GGG CAA GGA G 3'
 15 ZC1892 5' AGC TTG AGC GT 3'
 ZC1893 5' CTA GAC GCT CA 3'
 ZC1894 5' AGC TTC CAG TTC TTC GGC CTC
 ATG TCA GTT CTT CGG CCT CAT GTG AT 3'
 ZC1895 5' CTA GAT CAC ATG AGG CCG
 20 AAG AAC TGA CAT GAG GCC GAA GAA CTG GA
 3'

The 3'-end of the cDNA was not isolated in the first cloning and was subsequently isolated by screening 6×10^5 phage of the oligo (dT)-primed cDNA library with a 630 bp Sst I-Eco RI fragment derived from the 3'-end of clone RP41. One isolate, designated OT91, was further analyzed by restriction enzyme mapping and DNA sequencing. This clone was found to comprise the 3'-end of the receptor coding region and 1986 bp of 3' untranslated sequence.

Clones RP51, RP41 and OT91 were ligated together to construct a full-length cDNA encoding the entire PDGF receptor. RP41 was digested with Acc I and Bam HI to isolate the 2.12 kb fragment. RP51 was digested with Eco RI and Acc I to isolate the 982 bp fragment. The 2.12 kb RP41 fragment and the 982 bp RP51 fragment were joined in a three-part ligation with pUC13, which had been linearized by digestion with Eco RI and Bam HI. The resultant plasmid was designated 51/41. Plasmid 51/41 was digested with Eco RI and Bam HI to isolate the 3 kb fragment comprising the partial PDGF receptor cDNA. OT91 was digested with Bam HI and Xba I to isolate the 1.4 kb fragment containing the 3' portion of the PDGF receptor cDNA. The Eco RI-Bam HI 51/41 fragment, the Bam HI-Xba I OT91 fragment was joined with and Eco RI-Xba I digested pUC13 were joined in a three-part ligation. The resultant plasmid was designated pR-RX1 (Figure 2).

Example 2

Construction of a SUC2 Signal Sequence-PDGF Receptor Fusion

To direct the PDGF receptor into the yeast secretory pathway, the PDGF receptor cDNA was joined to a sequence encoding the *Saccharomyces cerevisiae* SUC2 signal sequence. Oligonucleotides ZC1453 and ZC1454 (Table 1) were designed to form an adapter encoding the SUC2 secretory peptide flanked by a 5' Eco RI adhesive end and a 3' Bgl II adhesive end. ZC1453 and ZC1454 were annealed under conditions described by Maniatis et al. (ibid.). Plasmid pR-RX1 was digested with Bgl II and Sst II to isolate the 1.7 kb fragment comprising the PDGF receptor coding sequence from amino acids 28 to 596. Plasmid pR-RX1 was also cut with Sst II and Hind III to isolate the 1.7 kb fragment comprising the coding sequence from amino acids 597 through the translation termination codon and 124 bp of 3' untranslated DNA. The two 1.7 kb pR-RX1 fragments and the ZC1453/ZC1454 adapter were joined with pUC19, which had been linearized by digestion with Eco RI and Hind III. The resultant plasmid, comprising the SUC2 signal sequence fused in-frame with the PDGF receptor cDNA, was designated pBTL10 (Figure 2).

Example 3

Construction of pCBS22

The BAR1 gene product, Barrier, is an exported protein that has been shown to have three domains. When used in conjunction with a signal sequence (von Heinje, *Eur. J. Biochem.* 133: 17-21, 1983; J. Mol. Biol. 184: 99-105, 1985; *Nuc. Acids Res.* 14: 4683-4690, 1986), the third domain of Barrier protein has been shown to aid in the secretion of proteins into the medium (MacKay et al., U.S. Patent Application Serial No. 104,316).

The third domain of the BAR1 gene was joined to a sequence encoding the C-terminal portion of substance P (subP; Munro and Pelham, *EMBO J.* 3: 3087-3093, 1984). The presence of the substance P amino acids on the terminus of the fusion protein allowed the protein to be detected using commercially available anti-substance P antibodies. Plasmid pZV9 (deposited as a transformant in *E. coli* strain RR1, ATCC accession no. 53283), comprising the entire BAR1 coding region and its associated flanking regions, was cut with Sal I and Bam HI to isolate the 1.3 kb BAR1 fragment. This fragment was subcloned into pUC13, which had been

cut with Sal I and Bam HI, to generate the plasmid designated pZV17. Plasmid pZV17 was digested with Eco RI to remove the 3'-most 0.5 kb of the BAR1 coding region. The vector-BAR1 fragment was religated to create the plasmid designated pJH66 (Figure 3). Plasmid pJH66 was linearized with Eco RI and blunt-ended with DNA polymerase I (Klenow fragment). Kinased Bam HI linkers (5' CCG GAT CCG G 3') were added and excess linkers were removed by digestion with Bam HI before religation. The resultant plasmid was designated pSW8 (Figure 3).

Plasmid pSW81, comprising the TP11 promoter, the BAR1 coding region fused to the coding region of the C-terminal portion of substance P (Munro and Pelham, *EMBO J.* 3: 3087-3093, 1984) and the TP11 terminator, was derived from pSW8. Plasmid pSW8 was cut with Sal I and Bam HI to isolate the 824 bp fragment encoding amino acids 252 through 526 of BAR1. Plasmid pPM2, containing the synthetic oligonucleotide sequence encoding the dimer form of the C-terminal portion of substance P (subP) in M13mp8, was obtained from Hugh Pelham (MRC Laboratory of Molecular Biology, Cambridge, England). Plasmid pPM2 was linearized by digestion with Bam HI and Sal I and ligated with the 824 bp BAR1 fragment from pSW8. The resultant plasmid, pSW14, was digested with Sal I and Sma I to isolate the 871 bp BAR1-substance P fragment. Plasmid pSV16, comprising a fragment of BAR1 encoding amino acids 1 through 250, was cut with Xba I and Sal I to isolate the 787 bp BAR1 fragment. This fragment was ligated with the 871 bp BAR1-substance P fragment in a three-part ligation with pUC18 cut with Xba I and Sma I. The resultant plasmid, designated pSW15, was digested with Xba I and Sma I to isolate the 1.64 kb BAR1-substance P fragment. The ADH1 promoter was obtained from pRL029. Plasmid pRL029, comprising the ADH1 promoter and the BAR1 5' region encoding amino acids 1 to 33 in pUC18, was digested with Sph I and Xba I to isolate the 0.42 kb ADH1 promoter fragment. The TP11 terminator (Alber and Kawasaki, ibid.) was provided as a linearized fragment containing the TP11 terminator and pUC18 with a Klenow-filled Xba I end and an Sph I end. This fragment was ligated with the 0.42 kb ADH1 promoter fragment and the 1.64 kb BAR1-substance P fragment in a three-part ligation to produce plasmid pSW22.

The ADH1 promoter and the coding region of BAR1, from the translation initiation ATG through the Eco RV site present in pSW22, were removed by digestion with Hind III and Eco RV. The 3.9 kb vector fragment, comprising the 401 bp between the Eco RV and the Eco RI sites of the BAR1 gene fused to subP and the TP11 terminator, was isolated by gel electrophoresis. Oligonucleotide

ZC1478 (Table 1) was kinased and annealed with oligonucleotide ZC1479 (Table 1) using conditions described by Maniatis et al. (ibid.). The annealed oligonucleotides formed an adapter comprising a Hind III adhesive end and a polylinker encoding Bgl II, Sph I, Nru I and Eco RV restriction sites. The ZC1479/ZC1478 adapter was ligated with the gel-purified pSW22 fragment. The resultant plasmid was designated pCBS22 (Figure 3).

Example 4

Construction of pBTL13

In order to enhance the secretion of the receptor and to facilitate the identification of the secreted protein, a sequence encoding the third domain of BAR1 fused to the C-terminal amino acids of substance P was fused in frame with the 5' 1240 bp of the PDGF receptor. Plasmid pBTL10 (Example 2) was digested with Sph I and Sst I to isolate the 4 kb fragment comprising the SUC2 signal sequence, a portion of the PDGF receptor cDNA and the pUC19 vector sequences. Plasmid pCBS22 was digested with Sph I and Sst I to isolate the 1.2 kb fragment comprising the BAR1-subP fusion and the TPI1 terminator. These two fragments were ligated and the resultant plasmid was designated pBTL13 (Figure 4).

Example 5

Construction of an Expression Vector Encoding the Entire PDGF Receptor

The entire PDGF receptor was directed into the secretory pathway by fusing a SUC2 signal sequence to the 5' end of the PDGF receptor coding sequence. This fusion was placed behind the TPI1 promoter and inserted into the vector YEp13 for expression in yeast.

The TPI1 promoter was obtained from plasmid pTPIC10 (Alber and Kawasaki, J. Mol. Appl. Genet. 1: 410-434, 1982), and plasmid pFATPOT (Kawasaki and Bell, EP 171,142; ATCC 20699). Plasmid pTPIC10 was cut at the unique Kpn I site, the TPI1 coding region was removed with Bal-31 exonuclease, and an Eco RI linker (sequence: GGA ATT CC) was added to the 3' end of the promoter. Digestion with Bgl II and Eco RI yielded a TPI1

promoter fragment having Bgl II and Eco RI sticky ends. This fragment was then joined to plasmid YRp7' (Stinchcomb et al., Nature 282: 39-43, 1979) that had been cut with Bgl II and Eco RI (partial). The resulting plasmid, TE32, was cleaved with Eco RI (partial) and Bam HI to remove a portion of the tetracycline resistance gene. The linearized plasmid was then recircularized by the addition of an Eco RI-Bam HI linker to produce plasmid TEA32. Plasmid TEA32 was digested with Bgl II and Eco RI, and the 900 bp partial TPI1 promoter fragment was gel-purified. Plasmid pIC9H (Marsh et al., Gene 32:481-486, 1984) was cut with Bgl II and Eco RI and the vector fragment was gel purified. The TPI1 promoter fragment was then ligated to the linearized pIC9H and the mixture was used to transform E. coli RR1. Plasmid DNA was prepared and screened for the presence of a -900 bp Bgl II-Eco RI fragment. A correct plasmid was selected and designated pICTPIP.

The TPI1 promoter was then subcloned to place convenient restriction sites at its ends. Plasmid pIC7 (Marsh et al., ibid.) was digested with Eco RI, the fragment ends were blunted with DNA polymerase I (Klenow fragment), and the linear DNA was recircularized using T4 DNA ligase. The resulting plasmid was used to transform E. coli RR1. Plasmid DNA was prepared from the transformants and was screened for the loss of the Eco RI site. A plasmid having the correct restriction pattern was designated pIC7RI'. Plasmid pIC7RI' was digested with Hind III and Nar I, and the 2500 bp fragment was gel-purified. The partial TPI1 promoter fragment (ca. 900 bp) was removed from pICTPIP using Nar I and Sph I and was gel-purified. The remainder of the TPI1 promoter was obtained from plasmid pFATPOT by digesting the plasmid with Sph I and Hind III, and a 1750 bp fragment, which included a portion of the TPI1 promoter fragment from pICTPIP, and the fragment from pFATPOT were then combined in a triple ligation to produce pMVR1 (Figure 2).

The TPI1 promoter was then joined to the SUC2-PDGF receptor fusion. Plasmid pBTL10 (Example 2) was digested with Eco RI and Hind III to isolate the 3.4 kb fragment comprising the SUC2 signal sequence and the entire PDGF receptor coding region. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment and the fragment derived from pBTL10 were joined with YEp13, which had been linearized by digestion with Bam HI and Hind III, in a three-part ligation. The resultant plasmid was designated pBTL12 (Figure 2).

Example 6

Construction of an Expression Vector Encoding the 5' Extracellular Portion of the PDGF Receptor

The extracellular portion of the PDGF receptor was directed into the secretory pathway by fusing the coding sequence to the SUC2 signal sequence. This fusion was placed in an expression vector behind the TPI1 promoter. Plasmid pBTL10 (Example 2) was digested with Eco RI and Sph I to isolate the approximately 1.3 kb fragment comprising the SUC2 signal sequence and the PDGF receptor extracellular domain coding sequence. Plasmid pMVR1 (Example 5) was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment was joined with the fragment derived from pBTL10 and YEp13, which had been linearized by digestion with Bam HI and Sph I, in a three-part ligation. The resultant plasmid was designated pBTL11 (Figure 2).

Example 7

Construction of Yeast Expression Vectors pBTL14 and pBTL15, and The Expression of PDGF Receptor-BAR1-subP Fusions

A. Construction of pBTL14

The SUC2-PDGF-R fusion was joined with the third domain of BAR1 to enhance the secretion of the receptor, and the expression unit was cloned into a derivative of YEp13 termed pJH50. YEp13 was modified to destroy the Sal I site near the LEU2 gene. This was achieved by partially digesting YEp13 with Sal I followed by a complete digestion with Xho I. The 2.0 kb Xho I-Sal I fragment comprising the LEU2 gene and the 8.0 kb linear YEp13 vector fragment were isolated and ligated together. The ligation mixture was transformed into E. coli strain RR1. DNA was prepared from the transformants and was analyzed by digestion with Sal I and Xho I. A clone was isolated which showed a single Sal I site and an inactive Xho I site indicating that the LEU2 fragment had inserted in the opposite orientation relative to the parent plasmid YEp13. The plasmid was designated pJH50.

Referring to Figure 4, plasmid pBTL12 (Example 5) was digested with Sal I and Pst I to isolate the 2.15 kb fragment comprising 270 bp of

YEp13 vector sequence, the TPI1 promoter, the SUC2 signal sequence, and 927 bp of PDGF receptor cDNA. Plasmid pBTL13 (Example 4) was digested with Pst I and Hind III to isolate the 1.48 kb fragment comprising 313 bp of PDGF receptor cDNA, the BAR1-subP fusion and the TPI1 terminator. The fragments derived from pBTL12 and pBTL13 were joined with pJH50, which had been linearized by digestion with Hind III and Sal I, in a three-part ligation. The resultant plasmid was designated pBTL14.

B. Construction of pBTL15

Referring to Figure 5, a yeast expression vector was constructed comprising the TPI1 promoter, the SUC2 signal sequence, 1.45 kb of PDGF receptor cDNA sequence fused to the BAR1-subP fusion and the TPI1 terminator. Plasmid pBTL12 (Example 5) was digested with Sal I and Fsp I to isolate the 2.7 kb fragment comprising the TPI1 promoter, the SUC2 signal sequence, the PDGF-R coding sequence, and 270 bp of YEp13 vector sequence. Plasmid pBTL13 (Example 4) was digested with Nru I and Hind III to isolate the 1.4 kb fragment comprising the BAR1-subP fusion, the TPI1 terminator and 209 bp of 3' PDGF receptor cDNA sequence. The fragments derived from pBTL12 and pBTL13 were joined in a three-part ligation with pJH50, which had been linearized by digestion with Hind III and Sal I. The resultant plasmid was designated pBTL15.

C. Expression of PDGF-R-subP fusions from pBTL15 and pBTL15

Yeast expression vectors pBTL14 and pBTL15 were transformed into Saccharomyces cerevisiae strains ZY100 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1p-CAT) and ZY400 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1p-CAT Δmnn9::URA3). Transformations were carried out using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2).

Table 2

-LeuThrTrp Amino Acid Mixture

4 g adenine
3 g L-arginine
5 g L-aspartic acid

2 g L-histidine free base
6 g L-isoleucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
4 g uracil
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-LEUDS

20 g glucose
6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, MI)
0.6 g -LeuThrTrp Amino Acid Mixture
182.2 g sorbitol
18 g Agar

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40mg L-tryptophan. Pour plates and allow to solidify.

-LEUDS + sodium succinate, pH 6.5

20 g yeast nitrogen base
0.6 g -LeuTrpThr Amino Acid Mixture
182.2 g sorbitol
11.8 g succinic acid

Mix all ingredients in distilled water to a final volume of 1 liter. Adjust the pH of the solution to pH 6.5. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

Fermentation Medium

7 g/l yeast nitrogen base without amino acids or ammonium sulfate (DIFCO Laboratories)
0.6 g/l ammonium sulfate
0.5 M sorbitol
0.39 g/l adenine sulfate
0.01% polypropylene glycol

Mix all ingredients in distilled water. Autoclave 15 minutes. Add 80 ml 50% glucose for each liter of medium.

Super Synthetic -LEUD, pH 6.5 (liquid or solid

medium)

6.7 g yeast nitrogen base without amino acids or ammonium sulfate (Difco)
6 g ammonium sulfate
160 g adenine
0.6 g -LeuThrTrp Amino Acid Mixture
20 g glucose
11.8 g succinic acid

Mix all ingredients and add distilled water to a final volume of 800 ml. Adjust the pH of the solution to pH 6.4. Autoclave 15 minutes. For solid medium, add 18 g agar before autoclaving, autoclave and pour plates.

Super Synthetic-LEUDS, pH 6.4 (liquid or solid medium)

Use the same recipe as Super Synthetic-LEUD, pH 6.4, but add 182.2 g sorbitol before autoclaving.

YEPD

20 g glucose
20 g Bacto peptone (DIFCO Laboratories)
10 g Bacto yeast extract (DIFCO Laboratories)
18 g agar
4ml 1% adenine
8 ml 1% L-leucine

Mix all ingredients in distilled water, and bring to a final volume of 1 liter. Autoclave 25 minutes and pour plates.

The transformants were assayed for binding to an anti-PDGF receptor monoclonal antibody (PR7212) or an anti-substance P antibody by protein blot assay. ZY100[pBTL14] and ZY100-[pBTL15] transformants were grown overnight at 30°C in 5 ml Super Synthetic-LEUD, pH 6.4 (Table 2). ZY400[pBTL14] and ZY400[pBTL15] transformants were grown overnight at 30°C in 5 ml Super Synthetic-LEUDS, pH 6.4 (Table 2). The cultures were pelleted by centrifugation and the supernatants were assayed by protein blot assay using methods described in Example 13. Results of assays using PR7212 are shown in Table 3.

TABLE 3

Results of a protein blot probed with PR7212

Transformant:

| | |
|---------------|----|
| ZY100[pBTL14] | + |
| ZY400[pBTL14] | ++ |
| ZY100[pBTL15] | + |
| ZY400[pBTL15] | + |

Example 8

Construction of a SUC2-PDGF-R Fusion Comprising the Complete PDGF-R Extracellular Domain

A. Construction of pBTL22

The PDGF-R coding sequence present in pBTL10 was modified to delete the coding region 3' to the extracellular PDGF-R domain. As shown in Figure 6, plasmid pBTL10 was digested with Sph I and Bam HI and with Sph I and Sst II to isolate the 4.77 kb fragment and the 486 bp fragment, respectively. The 486 bp fragment was then digested with Sau 3A to isolate the .17 kb fragment. The .17 kb fragment and the 4.77 kb were joined by ligation. The resultant plasmid was designated pBTL21.

Plasmid pBTL21, containing a Bam HI site that was regenerated by the ligation of the Bam HI and Sau 3A sites, was digested with Hind III and Bam HI to isolate the 4.2 kb fragment. Synthetic oligonucleotides ZC1846 (Table 1) and ZC1847 (Table 1) were designed to form an adapter encoding the PDGF-R from the Sau 3A site after bp 1856 (Figure 1) to the end of the extracellular domain at 1958 bp (Figure 1), having a 5' Bam HI adhesive end that destroys the Bam HI site and a 3' Hind III adhesive end. Oligonucleotides ZC1846 and ZC1847 were annealed under conditions described by Maniatis et. al. (ibid.). The 4.2 pBTL21 fragment and the ZC1846/ZC1847 adapter were joined by ligation. The resultant plasmid, designated pBTL22, comprises the SUC2 signal sequence fused in proper reading frame to the extracellular domain of PDGF-R in the vector pUC19 (Figure 6).

B. Construction of pBTL28

An in-frame translation stop codon was inserted immediately after the coding region of the PDGF-R in pBTL22 using oligonucleotides ZC1892 (Table 1) and ZC1893 (Table 1). These oligonucleotides were designed to form an adapter encoding a stop codon in-frame with the PDGF-R coding sequence from pBTL22 flanked by a 5' Hind III adhesive end and a 3' Xba I adhesive end. Plasmid pBTL22 was

digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGF-R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pC7RI⁺ vector sequences and the TPI1 terminator. Oligonucleotides ZC1892 and ZC1893 were annealed to form a Hind III-Xba I adapter. The 1.6 kb SUC2-PDGF-R fragment, the 3.68 kb pMVR1 fragment and the ZC1892/ZC1893 adapter were joined in a three-part ligation. The resultant plasmid was designated pBTL27.

The expression unit present in pBTL27 was inserted into the yeast expression vector pJH50 by first digesting pJH50 with Bam HI and Sal I to isolate the 10.3 kb vector fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI and with Xho I and Eco RI to isolate the 0.9 kb TPI1 promoter fragment and the 1.65 kb fragment, respectively. The 10.3 kb pJH50 vector fragment, the 0.9 kb TPI1 promoter fragment and 1.65 kb fragment were joined in a three-part ligation. The resultant plasmid was designated pBTL28.

C. Construction of Plasmid pBTL30

The PDGF-R coding sequence present in plasmid pBTL22 was modified to encode the twelve C-terminal amino acids of substance P and an in-frame stop codon. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGF-R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pC7RI⁺ and the TPI1 terminator. Synthetic oligonucleotides ZC1894 (Table 1) and ZC1895 (Table 1) were annealed to form an adapter containing the codons for the twelve C-terminal amino acids of substance P followed by an in-frame stop codon and flanked on the 5' end with a Hind III adhesive end and on the 3' end with an Xba I adhesive end. The ZC1894/ZC1895 adapter, the 1.6 kb SUC2-PDGF-R fragment and the pMVR1 fragment were joined in a three-part ligation. The resultant plasmid, designated pBTL29, was digested with Eco RI and Xho I to isolate the 1.69 kb SUC2-PDGF-R-subP-TPI1 terminator fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. Plasmid pJH50 was digested with Bam HI and Sal I to isolate the 10.3 kb vector fragment. The 1.69 kb pBTL29 fragment, the 0.9 kb TPI1 promoter fragment and the 10.3 kb vector fragment were joined in a three-part ligation. The resulting plasmid was designated pBTL30.

Example 9

Construction and Expression of a SUC2-PDGF-R-IgG Hinge Expression Vector

An expression unit comprising the TPI1 promoter, the SUC2 signal sequence, the PDGF-R extracellular domain, an immunoglobulin hinge region and the TPI1 terminator was constructed. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.56 kb fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.7 kb fragment, comprising the TPI1 promoter, pIC7RI⁺ vector sequences and the TPI1 terminator. Oligonucleotides ZC1776 (Table 1) and ZC1777 (Table 1) were designed to form, when annealed, an adapter encoding an immunoglobulin hinge region with a 5' Hind III adhesive end and a 3' Xba I adhesive end. Oligonucleotides ZC1776 and ZC1777 were annealed under conditions described by Maniatis et al. (ibid.). The 1.56 kb pBTL22 fragment, the 3.7 kb fragment and the ZC1776/ZC1777 adapter were joined in a three-part ligation, resulting in plasmid pBTL24.

The expression unit of pBTL24, comprising the TPI1 promoter, SUC2 signal sequence, PDGF-R extracellular domain sequence, hinge region sequence, and TPI1 terminator, was inserted into pJH50. Plasmid pBTL24 was digested with Xho I and Hind III to isolate the 2.4 kb expression unit. Plasmid pJH50 was digested with Hind III and Sal I to isolate the 9.95 kb fragment. The 2.4 kb pBTL24 fragment and 9.95 kb pJH50 vector fragment were joined by ligation. The resultant plasmid was designated pBTL25.

Plasmid pBTL25 was transformed into *Saccharomyces cerevisiae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). The transformants were tested for their ability to bind the anti-PDGF-R monoclonal antibody PR7212 using the colony assay method described in Example 13. Plasmid pBTL25 transformants were patched onto nitrocellulose filters that had been wetted and supported by YEPD solid medium. Antibody PR7212 was found to bind to the PDGF-R-IgG Hinge fusion secreted by ZY400[pBTL25] transformants.

Example 10

Construction and Expression of a SUC2 signal sequence-PDGF-R Extracellular Domain-SUC2 Fusion

As shown in Figure 6, an expression unit comprising the TPI1 promoter, SUC2 signal sequence, PDGF-R extracellular domain sequence, and SUC2 coding sequence was constructed as follows. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGF-R fragment. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The SUC2 coding region was obtained from pJH40. Plasmid pJH40 was constructed by inserting the 2.0 kb Hind III-Hind III SUC2 fragment from pRB58 (Carlson et al., Cell 28:145-154, 1982) into the Hind III site of pUC19 followed by the destruction of the Hind III site 3' to the coding region. Plasmid pJH40 was digested with Hind III and Sal I to isolate the 2.0 kb SUC2 coding sequence. Plasmid pJH50 was digested with Sal I and Bam HI to isolate the 10.3 kb vector fragment. The 0.9 kb Bgl II-Eco RI TPI1 promoter fragment, the 1.6 kb Eco RI-Hind III SUC2-PDGF-R, the 2.0 kb Hind III-Sal I SUC2 fragment and the 10.3 kb Bam HI-Sal I vector fragment were joined in a four-part ligation. The resultant plasmid was designated pBTL26 (Figure 6).

Plasmid pBTL26 was transformed into *Saccharomyces cerevisiae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). ZY400 transformants (ZY400[pBTL26]) were assayed by protein blot (Example 13), colony blot (Example 13) and competition assay.

Protein blot assays were carried out on ZY400-[pBTL26] and ZY400[pJH50] (control) transformants that had been grown in flasks. Two hundred-fifty μ l of a 5 ml overnight cultures of ZY400-[pBTL26] in -LEUDS + sodium succinate, pH 6.5 (Table 2) were inoculated into 50 ml of -LEUDS + sodium succinate, pH 6.5. The cultures were incubated for 35 hours in an airbath shaker at 30°C. The culture supernatants were harvested by centrifugation. The culture supernatants were assayed as described in Example 13 and were found to bind pR7212 antibody.

Colony assays were carried out on ZY400-[pBTL26] transformants. ZY400[pBTL26] transformants were patched onto wetted nitrocellulose filters that were supported on a YEPD plate. The colony assay carried out as described in Example 13.A. showed that ZY400[pBTL26] antibodies bound PR7212 antibodies.

Competition binding assays were carried out on ZY400[pBTL26] and ZY400[pJH50] transformants. The transformants were grown in 2 liters of fermentation medium (Table 2) in a New Brunswick Bioflo2 (New Brunswick, Philadelphia, PA) with continuous pH control at pH 6.4. The cultures were adjusted to pH 7.5 immediately prior to harvesting.

Culture supernatants were concentrated in an Amicon concentrator (Amicon, San Francisco, CA) using an Amicon 10⁴ mw spiral filter cartridge. The concentrated supernatants were further concentrated using Amicon Centriprep 10's. Fifteen ml of the concentrated supernatant samples were added to the Centripreps, and the Centripreps were spun in a Beckman GRP centrifuge (Beckman Instruments Inc., Carlsbad, CA) at setting 5 for a total of 60 minutes. The concentrates were removed from the Centriprep and were assayed in the competition assay.

The competition binding assay measured the amount of ¹²⁵I-PDGF left to bind to fetal foreskin fibroblast cells after preincubation with the concentrate containing the PDGF-R-SUC2 fusion protein. The concentrate was serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-AA, PDGF-BB or PDGF-AB, and the mixtures were incubated for two hours at room temperature. Three hundred µg of unlabelled PDGF-BB was added to each sample mixture. The sample mixtures were added to 24 well plates containing confluent fetal foreskin fibroblast cells (AG1523, available from the Human Genetic Mutant Cell Repository, Camden, NJ). The cells were incubated with the mixture for four hours at 4°C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held at 4°C (PBS; Sigma, St. Louis, Mo.). Five hundred µl of PBS + 1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the PDGF-R-SUC2 fusion protein was able to competitively bind all the isoforms of PDGF.

Table 4

Binding Medium

500 ml Ham's F-12 medium
12 ml 1M HEPES, pH 7.4
5 ml 100x PSN (Penicillin/Streptomycin/Neomycin, Gibco)
1 gm rabbit serum albumin

Western Transfer Buffer

25 mM Tris, pH 8.3
19 mM glycine, pH 8.3
20% methanol

Western Buffer A

5 50 ml 1 M Tris, pH 7.4
20 ml 0.25 mM EDTA, pH 7.0
5 ml 10% NP-40
37.5 ml 4 M NaCl
2.5 g gelatin

10

The Tris, EDTA, NP-40 and NaCl were diluted to a final volume of one liter with distilled water. The gelatin was added to 300 ml of this solution and the solution was heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the remainder of the first solution and stirred at 4°C until cool. The buffer was stored at 4°C.

15

20

Western Buffer B

50 ml 1 M Tris, pH 7.4
20 ml 0.25 M EDTA, pH 7.0
25 5 ml 10% NP-40
58.4 g NaCl
2.5 g gelatin
4 g N-lauroyl sarcosine

30

The Tris, EDTA, NP-40, and the NaCl were mixed and diluted to a final volume of one liter. The gelatin was added to 300 ml of this solution and heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the original solution and the N-lauroyl sarcosine was added. The final mixture was stirred at 4°C until the solids were completely dissolved. This buffer was stored at 4°C.

35

40

2x Loading Buffer

36 ml 0.5 M Tris-HCl, pH 6.8
16 ml glycerol
45 16 ml 20% SDS
4 ml 0.5 Bromphenol Blue in 0.5 M Tris-HCl, pH 6.8

50

Mix all ingredients. Immediately before use, add 100 µl β-mercaptoethanol to each 900 µl dye mix

Example 11

55

**Construction and Expression of PDGF Receptor
Analog From BHK cells**

19

A. Construction of pBTL114 and pBTL115

The portions of the PDGF receptor extracellular domain present in pBTL14 and pBTL15 were placed in a mammalian expression vector. Plasmids pBTL14 and pBTL15 were digested with Eco RI to isolate the 1695 bp and 1905 bp SUC2 signal-PDGF-R-BAR1 fragments, respectively. The 1695 bp fragment and the 1905 bp fragment were each ligated to Zem229R that had been linearized by digestion with Eco RI. The mammalian expression vector Zem229R is a pUC18-based expression vector containing a unique Eco RI site for insertion of foreign DNA between the mouse metallothionein-I promoter and the SV40 transcription terminator. Zem229R also contains an expression unit of the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 transcription terminator.

The ligation mixtures were transformed into *E. coli* strain RR1. Plasmid DNA was prepared and the plasmids were subjected to restriction enzyme analysis. A plasmid having the 1695 bp pBTL14 fragment inserted into Zem229R in the correct orientation was designated pBTL114 (Figure 9). A plasmid having the 1905 bp pBTL15 fragment inserted into Zem229R in the correct orientation was designated pBTL115 (Figure 9).

B. Expression of pBTL114 and pBTL115 from tk⁻ts13 BHK cells

Plasmids pBTL114 and pBTL115 were each transfected into tk⁻ts13 cells using the calcium phosphate precipitation (essentially as described by Graham and van der Eb, *J. Gen. Virol.* 36:59-72, 1977). The transfected cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1x PSN antibiotic mix (Gibco 600-5640), 2.0 mM L-glutamine. The cells were selected in 250 nM methotrexate (MTX) for 14 days, and the resulting colonies were screened by the immunofilter assay (McCracken and Brown, *Biotecniques*, 82-87, March/April 1984). Plates were rinsed with PBS or No Serum medium (DMEM plus 1x PSN antibiotic mix). Teflon® mesh (Spectrum Medical Industries, Los Angeles, CA) was then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After six hours incubation at 37°C, filters were removed and placed in Western buffer A (Table 4) overnight at room temperature. The filters were developed using the antibody PR7212 and the procedure described in Example 13. The filters showed that

conditioned media from pBTL114 transfected and pBTL115 transfected BHK cells bound the PR7212 antibody indicating the presence of secreted PDGF-R.

Example 12

Construction and Expression of PDGF Receptor Analogs From Cultured Mouse Myeloma Cells

A. Construction of pICμPRE8

The immunoglobulin μ heavy chain promoter and enhancer were subcloned into pIC19H to provide a unique Hind III site 3' to the promoter. Plasmid p μ (Grosschedl and Baltimore, *Cell* 41:885-897, 1985) was digested with Sal I and Eco RI to isolate the 3.1 kb fragment comprising the μ promoter. Plasmid pIC19H was linearized by digestion with Eco RI and Xho I. The μ promoter fragment and the linearized pIC19H vector fragment were joined by ligation. The resultant plasmid, designated pIC μ 3, was digested with Ava II to isolate the 700 bp promoter fragment. The 700 bp fragment was blunt-ended by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. Plasmid pIC19H was linearized by digestion with Xho I, and the adhesive ends were filled in by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. The blunt-ended Ava II fragment was ligated with the blunt-ended, linearized pIC19H, and the ligation mixture was transformed into *E. coli* JM83. Plasmid DNA was prepared from the transformants and was analyzed by restriction digest. A plasmid with a Bgl II site 5' to the promoter was designated pIC μ pR1(-). Plasmid pICPR1(-) was digested with Hind III and Bgl II to isolate the 700 bp μ promoter fragment. Plasmid pIC μ 19RI was linearized by digestion with Hind III and Bam HI. The 700 bp promoter fragment was joined with the linearized pIC19RI by ligation. The resultant plasmid, designated pIC μ PR7, comprised the μ promoter with a unique Sma I site 5' to the promoter and a unique Hind III site 3' to the promoter.

The immunoglobulin heavy chain μ enhancer was inserted into the unique Sma I site to generate plasmid pIC μ PRE8. Plasmid pJ4 (obtained from F. Blattner, Univ. Wisconsin, Madison, Wisconsin), comprising the 1.5 kb Hind III-Eco RI μ enhancer fragment in the vector pAT153 (Amersham, Arlington Heights, IL), was digested with Hind III and

Eco RI to isolate the 1.5 kb enhancer fragment. The adhesive ends of the enhancer fragment were filled in by treatment with T4 DNA polymerase and deoxynucleotide triphosphates. The blunt-ended fragment and pICμPR7, which had been linearized by digestion with Sma I, were joined by ligation. The ligation mixture was transformed into *E. coli* RR1. Plasmid DNA was prepared from the transformants, and the plasmids were analyzed by restriction digests. A plasmid, comprising the μ enhancer in the proper orientation relative to the μ promoter, was designated pICμPRE8 (Figure 7).

B. Construction of pSDL114

The DNA sequence encoding the extracellular domain of the PDGF receptor was joined with the DNA sequence encoding the human immunoglobulin light chain constant region. The PDGF extracellular domain was obtained from mpBTL22, which comprised the Eco RI-Hind III fragment from pBTL22 (Example 8.A.) cloned into Eco RI-Hind III cut M13mp18. Single stranded DNA was prepared from a mpBTL22 phage clone, and the DNA was subjected to *in vitro* mutagenesis using the oligonucleotide ZC1886 (Table 1) and the method described by Kunkel (Proc. Natl. Acad. Sci. USA 82:488-492, 1985). A phage clone, comprising the mutagenized PDGF-R with a donor splice site (5' splice site) at the 3' end of the PDGF-R extracellular domain, was designated pBTLR-HX (Figure 7).

The native PDGF-R signal sequence was obtained from pPR5. Plasmid pPR5, comprising 738 bp of 5' coding sequence with an Eco RI site immediately 5' to the translation initiation codon, was constructed from by *in vitro* mutagenesis of the PDGF-R cDNA fragment from RP51 (Example 1). Replicative form DNA of RP51 was digested with Eco RI to isolate the 1.09 kb PDGF-R fragment. The PDGF-R fragment was cloned into the Eco RI site of M13mp18. Single stranded template DNA was prepared from a phage clone containing the PDGF-R fragment in the proper orientation. The template DNA was subjected to *in vitro* mutagenesis using oligonucleotide ZC1380 (Table 1) and the method described by Zoller and Smith (Meth. Enzymol. 100:468-500, 1983). The mutagenesis resulted in the placement of an Eco RI site immediately 5' to the translation initiation codon. Mutagenized phage clones were analyzed by dideoxy sequence analysis. A phage clone containing the ZC1380 mutation was selected, and replicative form (Rf) DNA was prepared from the phage clone. The Rf DNA was digested with Eco RI and Acc I to isolate the .63 kb fragment. Plasmid pR-RX1 (Example 1) was digested with Acc I

and Eco RI to isolate the 3.7 kb fragment. The .63 kb fragment and the 3.7 kb fragment were joined by ligation resulting in plasmid pPR5 (Figure 7).

As shown in Figure 7, the PDGF-R signal peptide was obtained from plasmid pPR5 as and 1.4 kb Eco RI-Sph I fragment. Replicative form DNA from phage clone pBTLR-HX was digested with Sph I and Hind III to isolate the approximately 0.25 kb PDGF-R fragment. Plasmid pUC19 was linearized by digestion with Eco RI and Hind III. The 1.4 kb Eco RI-Sph I PDGF-R fragment, the 0.25 kb Sph I-Hind III fragment from pBTLR-HX and the Eco RI-Hind III cut pUC19 were joined in a three-part ligation. The resultant plasmid, pSDL110, was digested with Eco RI and Hind III to isolate the 1.65 kb PDGF-R fragment. Plasmid pICHuC_κ3.9.11 was used as the source of the kappa gene (Figure 7). Plasmid pICHuC_κ3.9.11 comprises the 1.1 kb Sph I-Hinc I fragment of a human kappa gene, which has been treated with DNA polymerase DNA I (Klenow Fragment) to fill in the Hinc II adhesive end, subcloned into Sph I-Hinc II cut pUC19. Plasmid pICHuC_κ3.9.11 was digested with Hinc III and Eco RI to isolate the 1.1 kb human kappa constant region gene. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGF-R fragment, the 1.1 kb kappa light chain constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL112, was digested with Bam HI and Cla I to isolate the 2.75 kb fragment. Plasmid μPRE8 was linearized with Bgl II and Cla I. The 2.75 kb fragment and the linearized μPRE8 were joined by ligation. The resultant plasmid was designated pSDL114 (Figure 7).

Plasmid pSDL114 was linearized by digestion with Cla I and was transfected into SP2/0-Ag14 (ATCC CRL 1581) by electroporation using the method essentially described by Neumann et al. (EMBO J. 1:841-845, 1982). Transfectants were selected in growth medium containing methotrexate.

Media from drug resistant clones were tested for the presence of secreted PDGF receptor analogs by immunoprecipitation. Approximately one million drug resistant transfectants were grown in DMEM medium lacking cysteine + 2% fetal calf serum for 18 hours at 37°C in the presence of 50 μCi ³⁵S-cysteine. Media was harvested from the labeled cells and 250 μl of the spent media was assayed for binding to the anti-PDGF receptor antibody PR7212. PR7212 diluted in PBS was added to the media to a final concentration of 2.5 μg per 250 μl spent media. Five μg of rabbit anti-mouse Ig diluted in PBS was added to the PR7212/media mixtures. The immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS). The immunocomplexes

were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at -70 °C. The results of the immunoprecipitation showed that the PDGF receptor analog secreted by the transfectants was bound by the anti-PDGF receptor antibody.

C. Cotransfection of pSDL114 with an immunoglobulin heavy chain

Plasmid pSDL114 was linearized by digestion with *Cla* I and was cotransfected with pG1-Neo, which encodes a neomycin resistance gene and a complete immunoglobulin heavy chain gene. The immunoglobulin heavy chain gene of pG1-Neo comprises a mouse/human chimeric immunoglobulin gene (Figure 9). The mouse immunoglobulin heavy chain gene isolated from a lambda genomic DNA library constructed from a murine hybridoma cell line, NR-ML-05 using oligonucleotide probes designed to span the VH/D/JH junctions. The human immunoglobulin heavy chain gene was isolated from a human genomic library and oligonucleotide probes designed to span the hinge exon. The mouse immunoglobulin heavy chain gene was isolated as a 5.3 kb *Sst* I-Hind III fragment from the original phage clone and the human gamma-1 C gene was obtained from the original phage clone as a 2.0 kb Hind III-Eco RI fragment. The chimeric gamma-1 C gene was created by joining the VH and CH fragments via the common Hind III site and incorporating them with the *E. coli* neomycin resistance gene into pIC19H to yield pG1M-Neo.

The linearized pSDL114 and pG1M-Neo were transfected into SP2/0-Ag14 cells by electroporation. The transfectants were selected in growth medium containing methotrexate and neomycin. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay.

The competition binding assay measured the amount of ¹²⁵I-PDGF left to bind to human dermal fibroblast cells after preincubation with the spent media from pSDL114-pG1-neo transfected cells. The media were serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-BB, and the mixtures were incubated for two hours at room temperature. Three hundred µg of unlabelled PDGF-BB was added to each sample mixture. The sample mixtures were added to 24 well plates containing confluent human dermal fibroblast cells. The cells were incubated with the mixture for four hours at 4 °C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held at 4 °C (PBS; Sigma, St.

Louis, Mo.). Five hundred µl of PBS + 1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the protein produced from pSDL114-pG1-neo transfected cells was able to competitively bind PDGF-BB.

D. Construction of pSDL113.

As shown in Figure 8, the DNA sequence encoding the extracellular domain of the PDGF receptor was joined with the DNA sequence encoding a human immunoglobulin heavy chain constant region joined to a hinge sequence. Plasmid pSDL110 was digested with *Eco* RI and *Hind* III to isolate the 1.65 kb PDGF-R fragment. Plasmid pICHuγ 1M was used as the source of the heavy chain constant region and hinge region. Plasmid pICHuγ 1M comprises the approximately 6 kb *Hind* III-Xho I fragment of a human gamma-1 C gene subcloned into pUC19 that had been linearized by digestion with *Hind* III and *Sall*. Plasmid pICHuγ 1M was digested with *Hind* III and *Eco* RI to isolate the 6 kb kb fragment encoding the human heavy chain constant region. Plasmid pIC19H was linearized by digestion with *Eco* RI. The 1.65 kb PDGF-R fragment, the 6 kb heavy chain constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL111, was digested with *Bam* HI to isolate the 7.7 kb fragment. Plasmid pµPRE8 was linearized with *Bgl* II and was treated with calf intestinal phosphatase to prevent self-ligation. The 7.7 kb fragment and the linearized pµPRE8 were joined by ligation. A plasmid containing the insert in the proper orientation was designated pSDL113 (Figure 8).

E. Cotransfection of pSDL113 with pICφ5V_HHuC_κ-Neo

Plasmid pSDL113 is linearized by digestion with *Cla* I and is cotransfected with pICφ5V_HHuC_κ-Neo, which encodes a neomycin resistance gene and mouse/human chimeric immunoglobulin gene. The mouse immunoglobulin light chain gene isolated from a lambda genomic DNA library constructed from a murine hybridoma cell line, NR-ML-05 using oligonucleotide probes designed to span the VL/JL junction. The human Immunoglobulin light chain gene was isolated from a human genomic library and an oligonucleotide probe encoding a published human kappa C gene (Hieter et al., *Cell* 22:197-207, 1980). The mouse immunoglobulin heavy chain gene was subcloned

from the original mouse genomic phage clone into pIC19R as a 3 kb Xba I-Hinc II fragment. The human kappa C gene was subcloned from the original human genomic phage clone into pUC19 as a 2.0 kb Hind III-Eco RI fragment. The chimeric kappa gene was created by joining the mouse kappa and human kappa genes via the common Sph I site and incorporating them with the *E. coli* neomycin resistance gene into pIC19H to yield pIC ϕ 5V_kHuC_k-Neo.

The linearized pSDL113 and pIC ϕ 5V_kHuC_k-Neo are transfected into SP2/0-Ag14 cells by electroporation. The transfectants are selected in growth medium containing methotrexate and neomycin. Media from drug-resistant clones are tested for their ability to bind PDGF in a competition binding assay.

F. Cotransfection of pSDL113 and pSDL114

Plasmids pSDL113 and pSDL114 are linearized by digestion with Cla I. The linearized plasmids p416, comprising a DHFR expression unit, and are cotransfected by electroporation. Transfected cells are selected in growth medium containing methotrexate. Media from drug resistant colonies are tested for ligand binding.

Example 13

Assay Methods

A. Preparation of Nitrocellulose Filters for Colony Assay

Colonies of transformants were tested for secretion of PDGF receptor analogs by first growing the cells on nitrocellulose filters that had been laid on top of solid growth medium. Nitrocellulose filters (Schleicher & Schuell, Keene, NH) were placed on top of solid growth medium and were allowed to be completely wetted. Test colonies were patched onto the wetted filters and were grown at 30°C for approximately 40 hours. The filters were then removed from the solid medium, and the cells were removed by four successive rinses with Western Transfer Buffer (Table 4). The nitrocellulose filters were soaked in Western Buffer A (Table 4) for one hour, at room temperature, on a shaking platform with two changes of buffer. Secreted PDGF-R analogs were visualized on the filters described below.

B. Preparation of Protein Blot Filters

A nitrocellulose filter was soaked in Western Buffer A (Table 4) without the gelatin and placed in a Minifold (Schleicher & Schuell, Keene, NH). Five ml of culture supernatant was added without dilution to the Minifold wells, and the liquid was allowed to pass through the nitrocellulose filter by gravity. The nitrocellulose filter was removed from the minifold and was soaked in Western Buffer A (Table 3) for one hour on a shaking platform at room temperature. The buffer was changed three times during the hour incubation.

C. Preparation of Western Blot Filters

The transformants were analyzed by Western blot, essentially as described by Towbin et al. (Proc. Natl. Acad. Sci. USA 76: 4350-4354, 1979) and Gordon et al. (U.S. Patent No. 4,452,901). Culture supernatants from appropriately grown transformants were diluted with three volumes of 95% ethanol. The ethanol mixtures were incubated overnight at -70°C. The precipitates were spun out of solution by centrifugation in an SS-24 rotor at 18,000 rpm for 20 minutes. The supernatants were discarded and the precipitate pellets were resuspended in 200 μ l of dH₂O. Two hundred μ l of 2x loading buffer (Table 4) was added to each sample, and the samples were incubated in a boiling water bath for 5 minutes.

The samples were electrophoresed in a 15% sodium dodecylsulfate polyacrylamide gel under non-reducing conditions. The proteins were electrophoretically transferred to nitrocellulose paper using conditions described by Towbin et al. (ibid.). The nitrocellulose filter was then incubated in Western Buffer A (Table 4) for 75 minutes at room temperature on a platform rocker.

D. Processing the Filters for Visualization with PR7212

Filters prepared as described above were screened for proteins which were bound by a PDGF receptor specific monoclonal antibody, designated PR7212. The filters were removed from the Western Buffer A (Table 4) and placed in sealed plastic bags containing a 10 ml solution comprising 10 μ g/ml PR7212 monoclonal antibody diluted in Western Buffer A. The filters were incubated on a rocking platform overnight at 4°C or for one hour at room temperature. Excess antibody was removed with three 15-minute washes with Western Buffer A on a shaking platform at room temperature.

Ten μ l biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) in 20 ml Western Buffer A was added to the filters. The filters were re-incubated for one hour at room temperature on a platform shaker, and unbound conjugated antibody was removed with three fifteen-minute washes with Western Buffer A.

The filters were pre-incubated for one hour at room temperature with a solution comprising 50 μ l Vectastain Reagent A (Vector Laboratories) in 10 ml of Western Buffer A that had been allowed to incubate at room temperature for 30 minutes before use. The filters were washed with one quick wash with distilled water followed by three 15-minute washes with Western Buffer B (Table 4) at room temperature. The Western Buffer B washes were followed by one wash with distilled water.

During the preceding wash step, the substrate reagent was prepared. Sixty mg of horseradish peroxidase reagent (Bio-Rad, Richmond, CA) was dissolved in 20 ml HPLC grade methanol. Ninety ml of distilled water was added to the dissolved peroxidase followed by 2.5 ml 2 M Tris, pH 7.4 and 3.8 ml 4 M NaCl. 100 μ l of 30% H_2O_2 was added just before use. The washed filters were incubated with 75 ml of substrate and incubated at room temperature for 10 minutes with vigorous shaking. After the 10 minute incubation, the buffer was changed, and the filters were incubated for an additional 10 minutes. The filters were then washed in distilled water for one hour at room temperature. Positives were scored as those samples which exhibited coloration.

E. Processing the Filters For Visualization with an Anti-Substance P Antibody

Filters prepared as described above were probed with an anti-substance P antibody. The filters were removed from the Western Buffer A and rinsed with Western transfer buffer, followed by a 5-minute wash in phosphate buffered saline (PBS, Sigma, St. Louis, MO). The filters were incubated with a 10 ml solution containing 0.5 M 1-ethyl-3-(3-dimethylamino) propyl carbodiimide (Sigma) in 1.0 M NH_4Cl for 40 minutes at room temperature. After incubation, the filters were washed three times, for 5 minutes per wash, in PBS. The filters were blocked with 2% powdered milk diluted in PBS.

The filters were then incubated with a rat anti-substance P monoclonal antibody. (Accurate Chemical & Scientific Corp., Westbury, NY). Ten μ l of the antibody was diluted in 10 ml of antibody solution (PBS containing 20% fetal calf serum and 0.5% Tween-20). The filters were incubated at room temperature for 1 hour. Unbound antibody

was removed with four 5-minute washes with PBS.

The filters were then incubated with a biotin-conjugated rabbit anti-rat peroxidase antibody (Cappel Laboratories, Melvern, PA). The conjugated antibody was diluted 1:1000 in 10 ml of antibody solution for 2 hours at room temperature. Excess conjugated antibody was removed with four 5-minute washes with PBS.

The filters were pre-incubated for 30 minutes at room temperature with a solution containing 50 μ l Vectastain Reagent A (Vector Laboratories) and 50 μ l Vectastain Reagent B (Vector Laboratories) in 10 ml of antibody solution that had been allowed to incubate for 30 minutes before use. Excess Vectastain reagents were removed by four 5-minute washes with PBS.

During the preceding wash step, the substrate reagent was prepared. 60 mg of horseradish peroxidase reagent (Bio-Rad) was dissolved in 25 ml of HPLC grade methanol. Approximately 100 ml of PBS and 200 μ l H_2O_2 were added just before use. The filters were incubated with the substrate reagent for 10 to 20 minutes. The substrate was removed by a vigorous washing distilled water.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

The features disclosed in the foregoing description, in the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

Claims

1. A method for producing a secreted human PDGF receptor analog, comprising:
introducing into a host cell a DNA construct capable of directing the expression and secretion of a human PDGF receptor analog, said DNA construct containing a transcriptional promoter operatively linked to a secretory signal sequence followed downstream in proper reading frame by a DNA sequence encoding at least a portion of the extracellular domain of a human PDGF receptor, said portion including a ligand-binding domain;
growing said host cell in an appropriate growth medium; and
isolating said PDGF receptor analog from said host cell.

2. The method of claim 1 wherein said DNA sequence encodes a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine,

number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531.

3. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a DNA construct capable of directing the expression and secretion of a biologically active peptide dimer, said DNA construct containing a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity fused to a dimerizing protein;

growing said host cell in an appropriate growth medium under conditions allowing the dimerization and secretion of the peptide dimer; and isolating said peptide dimer from said host cell.

4. The method of claim 1 or claim 3 wherein said host cell is a yeast cell carrying a defect in a gene whose product is required for the addition of outer chain oligosaccharide moieties to glycoproteins.

5. The method of claim 1 or claim 3 wherein said host cell is a yeast cell containing a genetic defect in the MNN9 gene or a disruption of the MNN9 gene.

6. A method according to claim 1 or claim 3 wherein said secretory signal sequence is selected from the group consisting of the MF α 1 pre-pro sequence, the PHO5 signal sequence, the BAR1 signal sequence, the SUC2 signal sequence, the PDGF receptor signal sequence and the mouse immunoglobulin V_H signal sequence.

7. The method of claim 3 wherein said dimerizing protein comprises at least a portion of a protein selected from the group consisting of an immunoglobulin light chain, an immunoglobulin heavy chain and yeast invertase, wherein said portion associates as a dimer in a covalent or a non-covalent manner.

8. The method of claim 3 wherein said dimerizing protein is selected from the group consisting of an immunoglobulin heavy chain hinge region and yeast invertase.

9. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to a dimerizing protein, said dimerizing protein comprising an immunoglobulin light chain constant region; introducing into said host cell a second DNA construct containing a transcriptional promoter oper-

atively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, C _{γ} 1, C _{γ} 2, C _{γ} 3, C _{γ} 4, and μ joined to an immunoglobulin hinge region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said biologically active peptide dimer; and

isolating said biologically active peptide dimer from said host cell.

10. A method for producing a secreted, biologically active peptide dimer, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a peptide requiring dimerization for biological activity joined to a dimerizing protein, said dimerizing protein comprising at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, C _{γ} 1, C _{γ} 2, C _{γ} 3, C _{γ} 4, and μ joined to an immunoglobulin heavy chain hinge region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least an immunoglobulin light chain constant region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said biologically active peptide dimer; and

isolating said biologically active peptide dimer from said host cell.

11. A method according to any of claims 3, 9 or 10 wherein said peptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441.

12. A method for producing a secreted, ligand-binding receptor analog, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding receptor analog joined to a dimerizing protein, said dimerizing protein comprising an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ joined to an immunoglobulin heavy chain hinge region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said ligand-binding receptor analog; and

isolating said ligand-binding receptor analog from said host cell.

13. A method for producing a secreted, ligand-binding receptor analog, comprising:

introducing into a host cell a first DNA construct containing a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding receptor analog joined to a dimerizing protein, said dimerizing protein comprising at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ joined to an immunoglobulin hinge region;

introducing into said host cell a second DNA construct containing a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding at least an immunoglobulin light chain constant region;

growing said host cell in an appropriate growth medium under conditions that allow the dimerization and secretion of said ligand-binding receptor analog; and

isolating said ligand-binding receptor analog from said host cell.

14. The method of claim 12 or claim 13 wherein said ligand-binding receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441.

15. A method according to any of claims 9, 10, 12 or 13 wherein said first and second secretory signal sequences are selected from the group consisting of the MFα1 pre-pro sequence, the PHO5 signal sequence, the BAR1 signal sequence, the SUC2 signal sequence, the PDGF receptor signal sequence and the mouse immunoglobulin V_H signal sequence.

16. A method according to any of claims 1, 3, 8, 9, 11 or 12 wherein the host cell is a yeast cell or a cultured mammalian cell.

17. A human PDGF receptor analog consisting essentially of the extracellular domain of a human PDGF receptor, said portion being capable of binding human PDGF or isoforms thereof.

18. The human PDGF receptor analog of claim 17 wherein said receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531.

19. A method for determining the presence of human PDGF or isoforms thereof in a biological sample, comprising:

incubating a polypeptide comprising a human PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of containing human PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and

detecting the presence of the receptor/ligand complexes as an indication of the presence of human PDGF or an isoform thereof.

20. The method of claim 19 wherein said human PDGF receptor analog comprises a polypeptide selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531, fused to a dimerizing protein.

21. The method of claim 19 wherein said dimerizing protein is selected from the group consisting of an immunoglobulin light chain constant region, an immunoglobulin heavy chain hinge region, at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, C_γ1, C_γ2, C_γ3, C_γ4, and μ joined to an immunoglobulin heavy chain hinge region and yeast invertase.

22. A pharmaceutical composition comprising a human PDGF receptor analog fused to a dimerizing protein in combination with a physiologically acceptable carrier or diluent.

23. The pharmaceutical composition of claim 22 wherein said PDGF receptor analog is selected from the group consisting of a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to methionine, number 441 and a polypeptide comprising the amino acid sequence of Figure 1 from isoleucine, number 29, to lysine, number 531.

24. A diagnostic composition comprising a polypeptide comprising a human PDGF receptor analog fused to a dimerizing protein, said polypeptide tagged with a label capable of providing a detectable signal.

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25. A method for purifying human PDGF of an isoform thereof from a sample comprising: immobilizing a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein on a substrate;

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contacting a sample containing human PDGF or an isoform thereof with the immobilized polypeptide under conditions such that the human PDGF or isoform thereof binds to the polypeptide; and eluting the human PDGF or isoform thereof from the polypeptide.

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FIG. 1

10 20 30 40 50 60 70
 CCCTCAGCCC TGCTGCCAG CACGAGCCTG TGCTGCCCTT GCCCAACGCA GACAGCCAGA CCCAGGGCGG
 80 90 100 110 120 130 140
 CCCCTCTGGC GGCTCTGCTC CTCCCGAAGG ATGCTTGGG AGTGAGGCGA AGCTGGGCGC TCCTCTCCCC
 150 160 170 180 190 200 210
 TACAGCAGCC CCCTTCTCTCC ATCCCTCTGT TCTCCTGAGC CTTCAGGAGC CTGCACCAGT CCTGCCCTGTC
 220 230 240 250 260 270 280
 CTTCTACTCA GCTGTTACCC ACTCTGGGAC CAGCAGTCTT TCTGATAACT GGGAGAGGGC AGTAAGGAGG
 290 300 310 320 330 340 350
 ACTTCCCTGGA GGGGGTGA CTCCAGAGCC TGGAACCTGT CCCACACCAG AAGCCATCAG CAGCAAGGAC
 359 368 377 386 395 404
 ACC ATG CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC GAG CTG CTG
 M R L P G A M P A L A L K G E L L 17
 413 422 431 440 449 458
 TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT CAG GGC CTG GTC GTC
 L L S L L L L L E P Q I S Q G L V V 35
 467 476 485 494 503 512
 ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC TTC GTT CTG ACC
 T P P G P E L V L N V S S T F V L T 53
 521 530 539 548 557 566
 TGC TCG GGT TCA GCT CCG GTG GTG TGG GAA CGG ATG TCC CAG GAG CCC CCA CAG
 C S G S A P V V W E R M S Q E P P Q 71

FIG. 1 CONT.

| | | | | | |
|---|-----------|-------|-------|-----|-----|
| 575 | 584 | 593 | 602 | 611 | 620 |
| GAA ATG GCC AAG GCC CAG GAT GGC ACC TTC TCC AGC GTG CTC ACA CTG ACC AAC | | | | | |
| E M A K A Q | D G T F S | V L T | L T N | | 89 |
| 629 | 638 | 647 | 656 | 665 | 674 |
| CTC ACT GGG CTA GAC ACG GGA GAA TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA | | | | | |
| L T G L D T T G E Y F C T H N D S R G | | | | | 107 |
| 683 | 692 | 701 | 710 | 719 | 728 |
| CTG GAG ACC GAT GAG CGG AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG | | | | | |
| L E T D E R K R L Y I F V P D P T V | | | | | 125 |
| 737 | 746 | 755 | 764 | 773 | 782 |
| GGC TTC CTC CCT AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA ACT | | | | | |
| G F L P N D A E E L F I F L T E I T | | | | | 143 |
| 791 | 800 | 809 | 818 | 827 | 836 |
| GAG ATC ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG ACA CTG CAC | | | | | |
| E I T I P C R V T D P Q L V V T L H | | | | | 161 |
| 845 | 854 | 863 | 872 | 881 | 890 |
| GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT CAC CAA CGT GGC TTT | | | | | |
| E K K G G D V A L P V V P Y D H Q R G F | | | | | 179 |
| 899 | 908 | 917 | 926 | 935 | 944 |
| TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC AAA ACC ACC ATT GGG GAC AGG | | | | | |
| S G I F E D R S Y I C K T T I G D R | | | | | 197 |

FIG. 1 CONT

| | | | | | |
|-------------------------------------|-------------------------------------|------|------|------|------|
| 953 | GAG GTG GAT TCT GAT GCC TAC TAT GTC | 971 | 980 | 989 | 998 |
| E V D S D A A Y Y V V | 962 | 971 | 980 | 989 | 998 |
| 215 | | | | | |
| 1007 | GTC TCT GTG AAC GCA GTG CAG ACT GTG | 1025 | 1034 | 1043 | 1052 |
| V S V N A V A V Q T V V | 1016 | 1025 | 1034 | 1043 | 1052 |
| 233 | | | | | |
| 1061 | ATG TGC ATT GTG ATC GGG AAT GAG GTG | 1079 | 1088 | 1097 | 1106 |
| M C I V I G N E V V | 1070 | 1079 | 1088 | 1097 | 1106 |
| 251 | | | | | |
| 1115 | AAA GAA AGT GGG CGG CTG GTG GAG CCG | 1133 | 1142 | 1151 | 1160 |
| K E S G R L V E P V | 1124 | 1133 | 1142 | 1151 | 1160 |
| 269 | | | | | |
| 1169 | TAC CAC ATC CGC TCC ATC CTG CAC ATC | 1187 | 1196 | 1205 | 1214 |
| Y H I R S I L H I P S A E L E | 1178 | 1187 | 1196 | 1205 | 1214 |
| 287 | | | | | |
| 1223 | ACC TAC ACC TGC AAT GTG ACG GAG AGT | 1241 | 1250 | 1259 | 1268 |
| T Y T C N V T E S S V N D H Q D | 1232 | 1241 | 1250 | 1259 | 1268 |
| 305 | | | | | |
| 1277 | ATC AAC ATC ACC GTG GTT GAG AGC GGC | 1295 | 1304 | 1313 | 1322 |
| I N I T V V E S G Y V R L L G E V G | 1286 | 1295 | 1304 | 1313 | 1322 |
| 323 | | | | | |
| 1331 | ACA CTA CAA TTT GCT GAG CTG CAT CGG | 1349 | 1358 | 1367 | 1376 |
| T L Q F A E L L H R S R T L Q V F E | 1340 | 1349 | 1358 | 1367 | 1376 |
| 341 | | | | | |

FIG. 1 CONT.

| | | | | | |
|---|---------------------------------------|------|------|------|------|
| 1385 | 1394 | 1403 | 1412 | 1421 | 1430 |
| GCC TAC CCA CCG CCC ACT GTC CTG TGG TTC AAA GAC AAC CGC ACC CTG GGC GAC | A Y P P P T V L W F K D N R T L G D | 359 | | | |
| 1439 | 1448 | 1457 | 1466 | 1475 | 1484 |
| TCC AGC GCT GGC GAA ATC GCC CTG TCC ACG CGC AAC GTG TCG GAG ACC CGG TAT | S S A G E I A L S T R N V S E T R Y | 377 | | | |
| 1493 | 1502 | 1511 | 1520 | 1529 | 1538 |
| GTG TCA GAG CTG ACA CTG GTT CGC GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC | V S E L T L V R V K V A E A G H Y T | 391 | | | |
| 1547 | 1556 | 1565 | 1574 | 1583 | 1592 |
| ATG CGG GCC TTC CAT GAG GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC | M R A F H E D A E V Q L S F Q L Q I | 413 | | | |
| 1601 | 1610 | 1619 | 1628 | 1637 | 1646 |
| AAT GTC CCT GTC CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGC GAA | N V P V R V L E L S E S H P D S G E | 431 | | | |
| 1655 | 1664 | 1673 | 1682 | 1691 | 1700 |
| CAG ACA GTC CGC TGT CGT GGC CGG GGC ATG CCC CAG CCG AAC ATC ATC TGG TCT | Q T V R C R G R G M P Q P N I I W S | 449 | | | |
| 1709 | 1718 | 1727 | 1736 | 1745 | 1754 |
| GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG CCC ACG CTG CTG GGG | A C R D L K R R C P R E L P P T L L G | 467 | | | |

FIG. 1 CONT.

| | | | | | |
|---|------|------|------|------|------|
| 1763 | 1772 | 1781 | 1790 | 1799 | 1808 |
| AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT AAC GTG ACG TAC TGG GAG GAG | | | | | |
| N S S E E E S Q L E T N V T Y W E E | | | | | 485 |
| 1817 | 1826 | 1835 | 1844 | 1853 | 1862 |
| GAG CAG GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG CAG CAC GTG GAT CGG CCA | | | | | |
| E Q E F E V V S T L R L Q H V D R P | | | | | 503 |
| 1871 | 1880 | 1889 | 1898 | 1907 | 1916 |
| CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC | | | | | |
| L S V R C T L L R N A V G Q D T Q E V | | | | | 521 |
| 1925 | 1934 | 1943 | 1952 | 1961 | 1970 |
| ATC GTG GTG CCA CAC TCC TTG CCC TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG | | | | | |
| I V V P H S L P F K V V V I S A I L | | | | | 539 |
| 1979 | 1988 | 1997 | 2006 | 2015 | 2024 |
| GCC CTG GTG GTG CTC ACC ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG | | | | | |
| A L V V L T I I S L I I L I M L W Q | | | | | 557 |
| 2033 | 2042 | 2051 | 2060 | 2069 | 2078 |
| AAG AAG CCA CGT TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT GAC | | | | | |
| K K P R Y E I R W K V I E S V S D | | | | | 575 |
| 2087 | 2096 | 2105 | 2114 | 2123 | 2132 |
| GGC CAT GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC TCC ACG TGG | | | | | |
| G H E Y I Y V D P M Q L P Y D S T W | | | | | 593 |
| 2141 | 2150 | 2159 | 2168 | 2177 | 2186 |
| GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC GGC TCT GGG GCC TTT | | | | | |
| E L P R D Q L V L G R T L L G S G A F | | | | | 611 |

FIG. 1 CONT.

| | | | | | |
|---|---------------------------------------|------|------|------|------|
| 2195 | 2204 | 2213 | 2222 | 2231 | 2240 |
| GGG CAG GTG GTG GAG GCC ACG GCT CAT GGC CTG AGC CAT TCT CAG GCC ACG ATG | G Q V V E A T A H G L S H S Q A T M | 629 | | | |
| 2249 | 2258 | 2267 | 2276 | 2285 | 2294 |
| AAA GTG GCC GTC AAG ATG CTT AAA TCC ACA GCC CGC AGC AGT GAG AAG CAA GCC | K V A V K M L K S T A R S S E K Q A | 647 | | | |
| 2303 | 2312 | 2321 | 2330 | 2339 | 2348 |
| CTT ATG TCG GAG CTG AAG ATC ATG AGT CAC CTT GGG CCC CAC CTG AAC GTG GTC | L M S E L K I M S H L G P H L N V V | 665 | | | |
| 2357 | 2366 | 2375 | 2384 | 2393 | 2402 |
| AAC CTG TTG GGG GCC TGC ACC AAA GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC | N L L G A C T K G G G P I Y I I T E Y | 683 | | | |
| 2411 | 2420 | 2429 | 2438 | 2447 | 2456 |
| TGC CGC TAC GGA GAC CTG GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG | C R Y G G D L V D Y L H R N K H T F L | 701 | | | |
| 2465 | 2474 | 2483 | 2492 | 2501 | 2510 |
| CAG CAC CAC TCC GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC AGC AAT GCT | Q H H S D K R R P P P S A E L Y S N A | 719 | | | |
| 2519 | 2528 | 2537 | 2546 | 2555 | 2564 |
| CTG CCC GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG GAG AGC GAC | L P V G L P L P S H V S L T G E S D | 737 | | | |

FIG. 1 CONT.

| | | | | | |
|---|---------------------------------------|------|------|------|------|
| 2573 | 2582 | 2591 | 2600 | 2609 | 2618 |
| GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC TAT GTG CCC ATG CTG | G G Y M D M S K D E S V D Y V P M L | | | | 755 |
| 2627 | 2636 | 2645 | 2654 | 2663 | 2672 |
| GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC GAG TCC TCC AAC TAC ATG GCC | D M K G G D V K Y A D I E S S N Y M A | | | | 773 |
| 2681 | 2690 | 2699 | 2708 | 2717 | 2726 |
| CCT TAC GAT AAC TAC GTT CCC TCT GCC CCT GAG AGG ACC TGC CGA GCA ACT TTG | P Y D N Y V P S A P E R T C R A T L | | | | 791 |
| 2735 | 2744 | 2753 | 2762 | 2771 | 2780 |
| ATC AAC GAG TCT CCA GTG CTA AGC TAC ATG GAC CTC GTG GGC TTC AGC TAC CAG | I N E S P V L S Y M D L V G F S Y Q | | | | 809 |
| 2789 | 2798 | 2807 | 2816 | 2825 | 2834 |
| GTG GCC AAT GGC ATG GAG TTT CTG GCC TCC AAC AAC TGC CTC CAC AGA GAC CTG | V A N G M E F L A S K N C V H R D L | | | | 827 |
| 2843 | 2852 | 2861 | 2870 | 2879 | 2888 |
| GGC GCT AGG AAC GTG CTC ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT | A A R N V L I C E G K L V K I C D F | | | | 845 |
| 2897 | 2906 | 2915 | 2924 | 2933 | 2942 |
| GGC CTG GCT CGA GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC ACC | G L A R D I M R D S N Y I S K G S T | | | | 863 |
| 2951 | 2960 | 2969 | 2978 | 2987 | 2996 |
| TTT TTG CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC CTC TAC ACC | F L P L K W M A P E S I F N S L Y T | | | | 881 |

FIG. 1 CONT.

| | | | | | |
|---|------|------|------|------|------|
| 3005 | 3014 | 3023 | 3032 | 3041 | 3050 |
| ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG GAG ATC TTC ACC TTG | | | | | |
| T L S D V W S F G I L L W E I F T L | | | | | 899 |
| 3059 | 3068 | 3077 | 3086 | 3095 | 3104 |
| GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC GAG CAG TTC TAC AAT GCC ATC | | | | | |
| G G T P Y P E L P M N E Q F Y N A I | | | | | 917 |
| 3113 | 3122 | 3131 | 3140 | 3149 | 3158 |
| AAA CGG GGT TAC CGC ATG GCC CAG CCT GCC CAT GGC TCC GAC GAG ATC TAT GAG | | | | | |
| K R G Y R M A Q P A H A S D E I Y E | | | | | 935 |
| 3167 | 3176 | 3185 | 3194 | 3203 | 3212 |
| ATC ATG CAG AAG TGC TGG GAA GAG AAG TTT GAG ATT CGG CCC TTC TCC CAG | | | | | |
| I M Q K C W E E K F E I R P P F S Q | | | | | 953 |
| 3221 | 3230 | 3239 | 3248 | 3257 | 3266 |
| CTG GTG CTG CTT CTC GAG AGA CTG TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG | | | | | |
| L V L L L E R L L G E G Y K K Y Q | | | | | 971 |
| 3275 | 3284 | 3293 | 3302 | 3311 | 3320 |
| CAG GTG GAT GAG GAG TTT CTG AGG AGT GAC CAC CCA GCC CTT CGG TCC CAG | | | | | |
| Q V D E E F L R S D H P A I L R S Q | | | | | 989 |
| 3329 | 3338 | 3347 | 3356 | 3365 | 3374 |
| GCC CGC TTG CCT GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC GTC | | | | | |
| A R L P G F H G L R S P L D T S S V | | | | | 1007 |

FIG. 1 CONT.

| | | | | | |
|---|------|------|------|------|------|
| 3383 | 3392 | 3401 | 3410 | 3419 | 3428 |
| CTC TAT ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC ATC CCC CTG | | | | | |
| L Y T A V Q P N E G D N D Y I I P L 1025 | | | | | |
| 3437 | 3446 | 3455 | 3464 | 3473 | 3482 |
| CCT GAC CCC AAA CCC GAG GTT GCT GAC GAG GGC CCA CTG GAG GGT TCC CCC AGC | | | | | |
| P D P K P E V A D E G P L E G S P S 1043 | | | | | |
| 3491 | 3500 | 3509 | 3518 | 3527 | 3536 |
| CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC TCC TCA ACC ATC TCC TGT GAC | | | | | |
| L A S S T L N E V N T S S T I S C D 1061 | | | | | |
| 3545 | 3554 | 3563 | 3572 | 3581 | 3590 |
| AGC CCC CTG GAG CCC CAG GAC GAA CCA GAG CCA GAG CCC CAG CTT GAG CTC CAG | | | | | |
| S P L E P Q D E P E P E P Q L E L Q 1079 | | | | | |
| 3599 | 3608 | 3617 | 3626 | 3635 | 3644 |
| GTG GAG CCG GAG CCA GAG CTG GAA CAG TTG CCG GAT TCG GGG TGC CCT GCG CCT | | | | | |
| V E P E P E L E Q L P D S G C P A P 1097 | | | | | |
| 3653 | 3662 | 3671 | 3684 | 3694 | 3704 |
| CGG GCG GAA GCA GAG GAT AGC TTC CTG TAG GGGCTGGCC CCTACCTGC CCTGCCCTGAA | | | | | |
| R A E A E D S F L . 1106 | | | | | |
| 3714 | 3724 | 3734 | 3744 | 3754 | 3764 |
| GCTCCCCC TGCCAGCACC CAGCATCTCC TGGCCTGGCC TGACCGGGCT TCCTGTCAGC CAGGCTGCCC | | | | | |
| 3784 | 3794 | 3804 | 3814 | 3824 | 3834 |
| TTATCAGCTG TCCCCCTCTG GAAGCTTTCT GCTCCTGACG TGTTGTGCCC CAAACCTGG GGCTGGCTTA | | | | | |
| 3854 | 3864 | 3874 | 3884 | 3894 | 3904 |
| GGAGGCAAGA AACTGCAGG GSCCGTGACC AGCCCTCTGC CTCCAGGGAG GCCAACTGAC TCTGAGCCAG | | | | | |
| 3914 | | | | | |

FIG. 1 CONT.

| | | | | | | |
|-------------|------------|------------|------------|------------|-------------|-------------|
| 3924 | 3934 | 3944 | 3954 | 3964 | 3974 | 3984 |
| GGTCCCCCA | GGGAACACAG | TTTCCCATTA | TGTAAGATGG | GAAAGTTAGG | CTTGATGACC | CAGAATCTAG |
| 3994 | 4004 | 4014 | 4024 | 4034 | 4044 | 4054 |
| GATTCTCTCC | CTGGCTGACA | GGTGGGAGAG | CCGAATCCCT | CCCTGGGAAG | ATTCTTGGAG | TTTACTGAGGT |
| 4064 | 4074 | 4084 | 4094 | 4104 | 4114 | 4124 |
| GGTAAATTAA | CTTTTCTG | TTCAGCCAGC | TACCCCTCAA | GGAATCATAG | CTCTCTCTCTC | GCACITTTTA |
| 4134 | 4144 | 4154 | 4164 | 4174 | 4184 | 4194 |
| TCCACCCAGG | AGCTAGGGA | GAGACCCTAG | CCTCCCTGGC | TGCTGGCTGA | GCTAGGGCCT | AGCCITGAGC |
| 4204 | 4214 | 4224 | 4234 | 4244 | 4254 | 4264 |
| AGTGTGCTCCT | CATCCAGAAG | AAAGCCAGTC | TCCTCCCTAT | GATGCCAGTC | CCTGCGTTC | CTGGCCCCGAG |
| 4274 | 4284 | 4294 | 4304 | 4314 | 4324 | 4334 |
| CTGGTCTGGG | GCCATTAGGC | AGCCTAATTA | ATGCTGGAGG | CTGAGCCAAG | TACAGGACAC | CCCCAGCCTG |
| 4344 | 4354 | 4364 | 4374 | 4384 | 4394 | 4404 |
| CAGCCCTTGC | CCAGGGCACT | TGGAGCACAC | GCAGCCATAG | CAAGTGCCTG | TGTCCCTGTC | CTTCAGGGCCC |
| 4414 | 4424 | 4434 | 4444 | 4454 | 4464 | 4474 |
| ATCAGTCCCTG | GGGCTTTTTC | TTTATCACCC | TCAGTCTTAA | TCCATCCACC | AGAGTCTAGA | AGGCCAGACG |
| 4484 | 4494 | 4504 | 4514 | 4524 | 4534 | 4544 |
| GGCCCCGCAT | CTGTGATGAG | AATGTAAATG | TGCCAGTGTG | GAGTGGCCAC | GTGTGTGTGC | CAGTATATGG |

FIG. 2

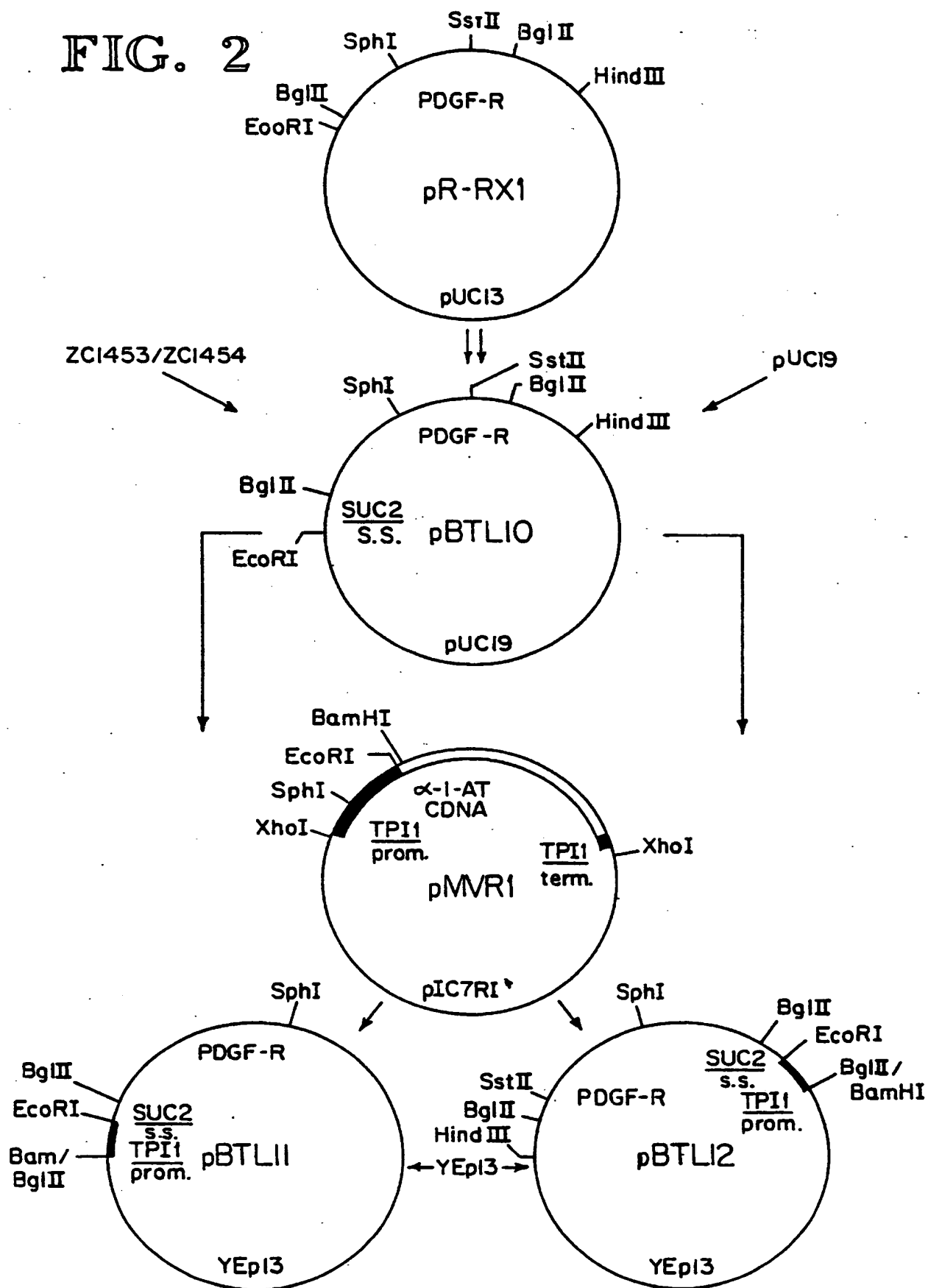


FIG. 3

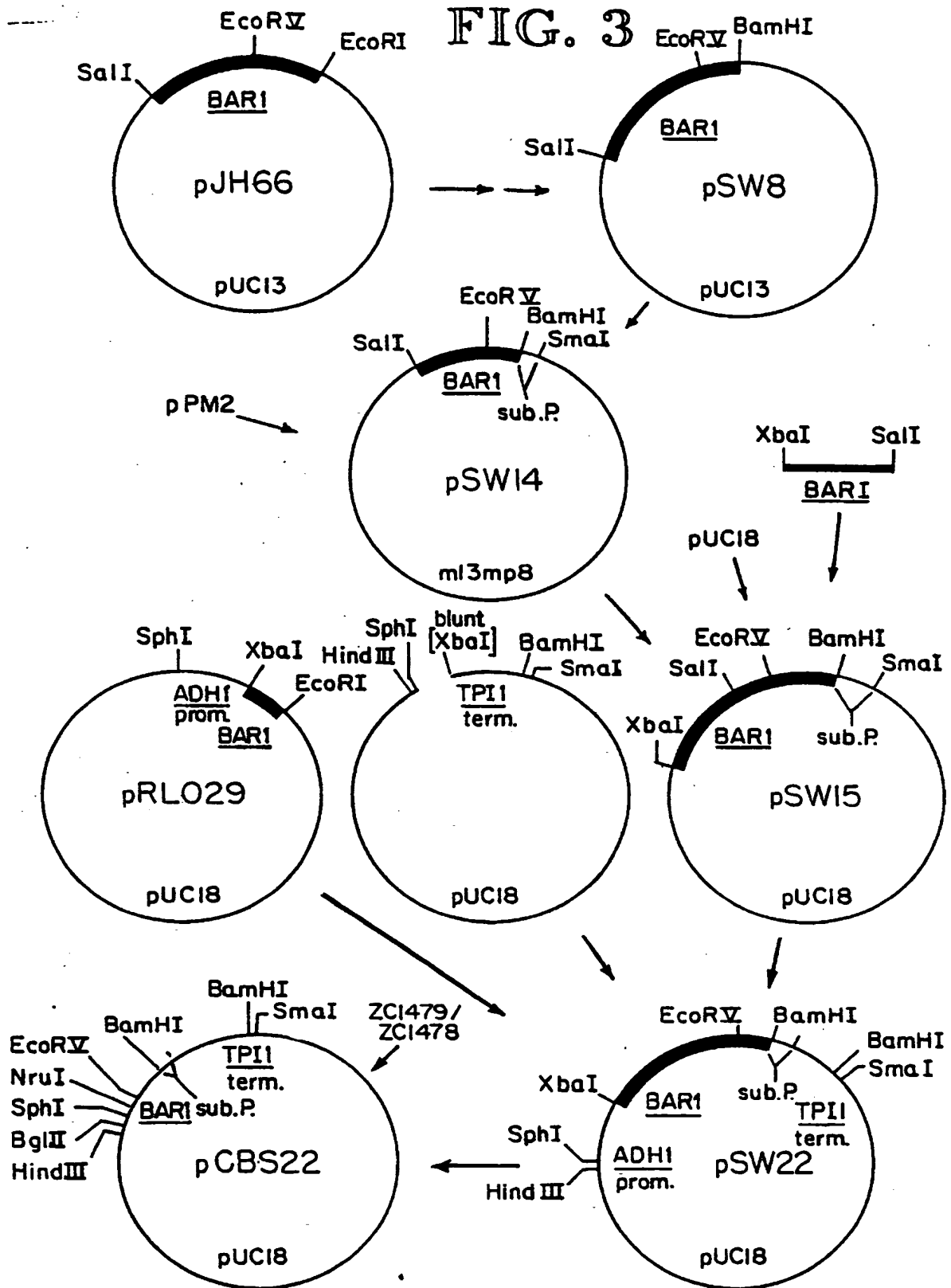


FIG. 4

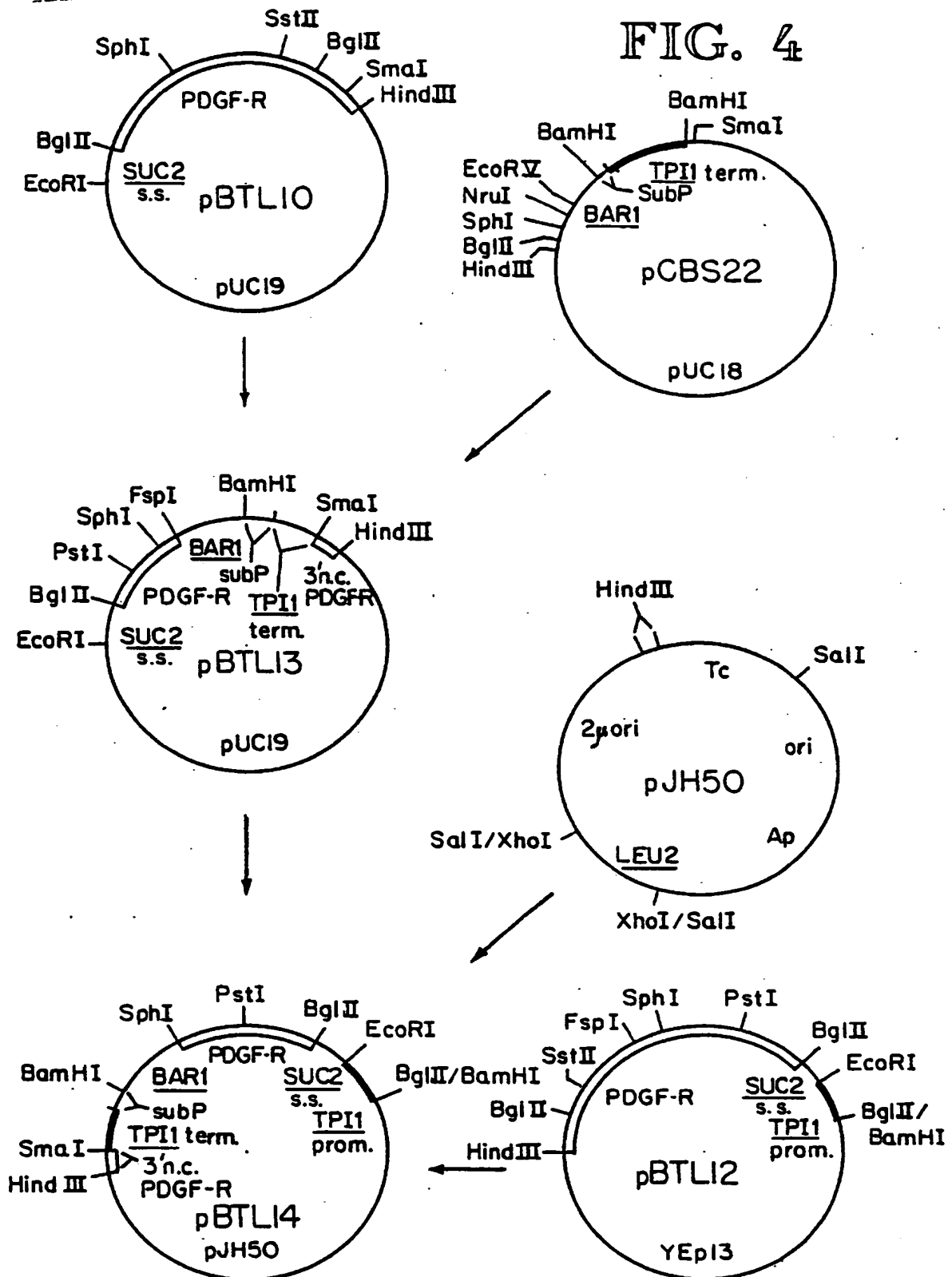


FIG. 5

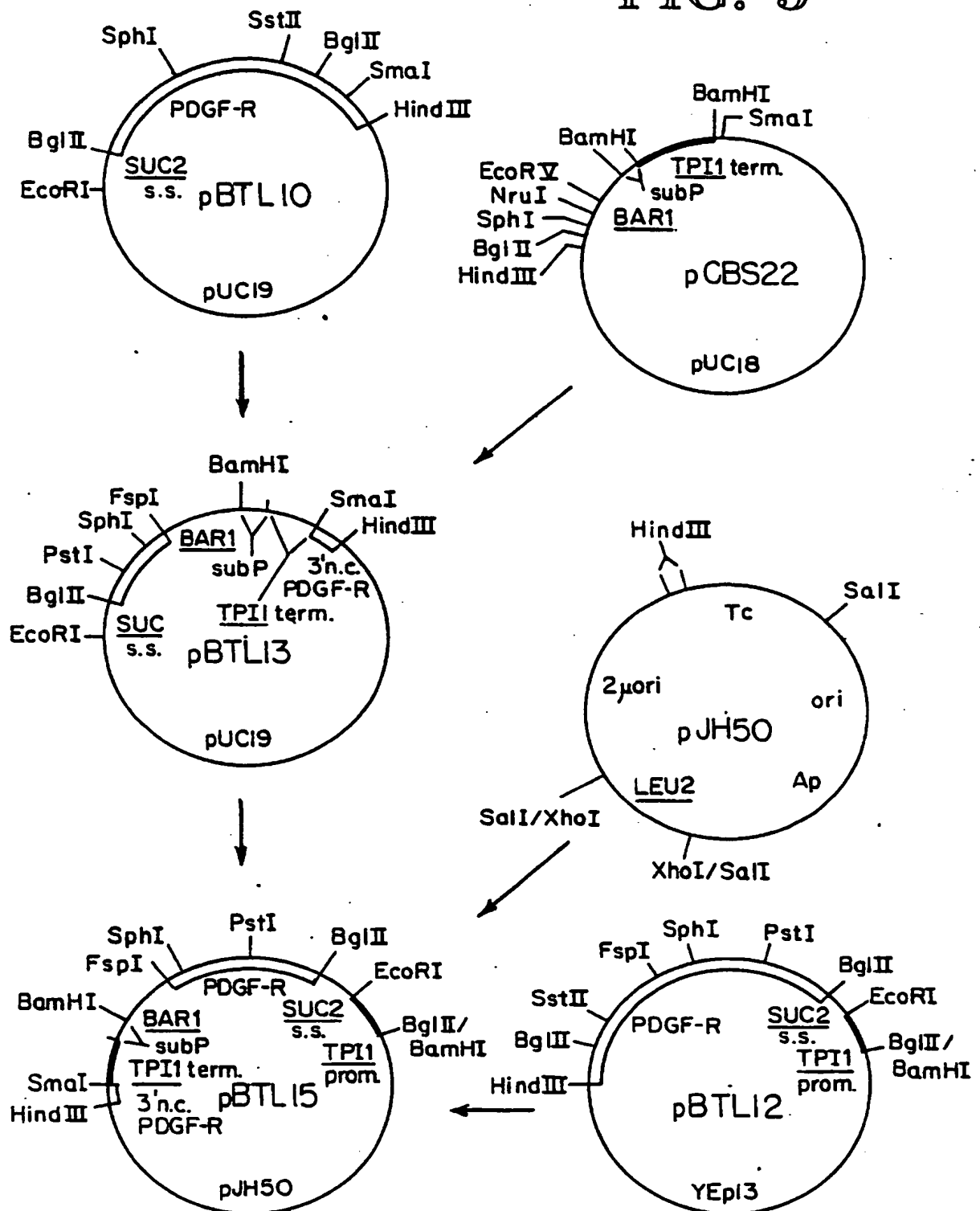


FIG. 6

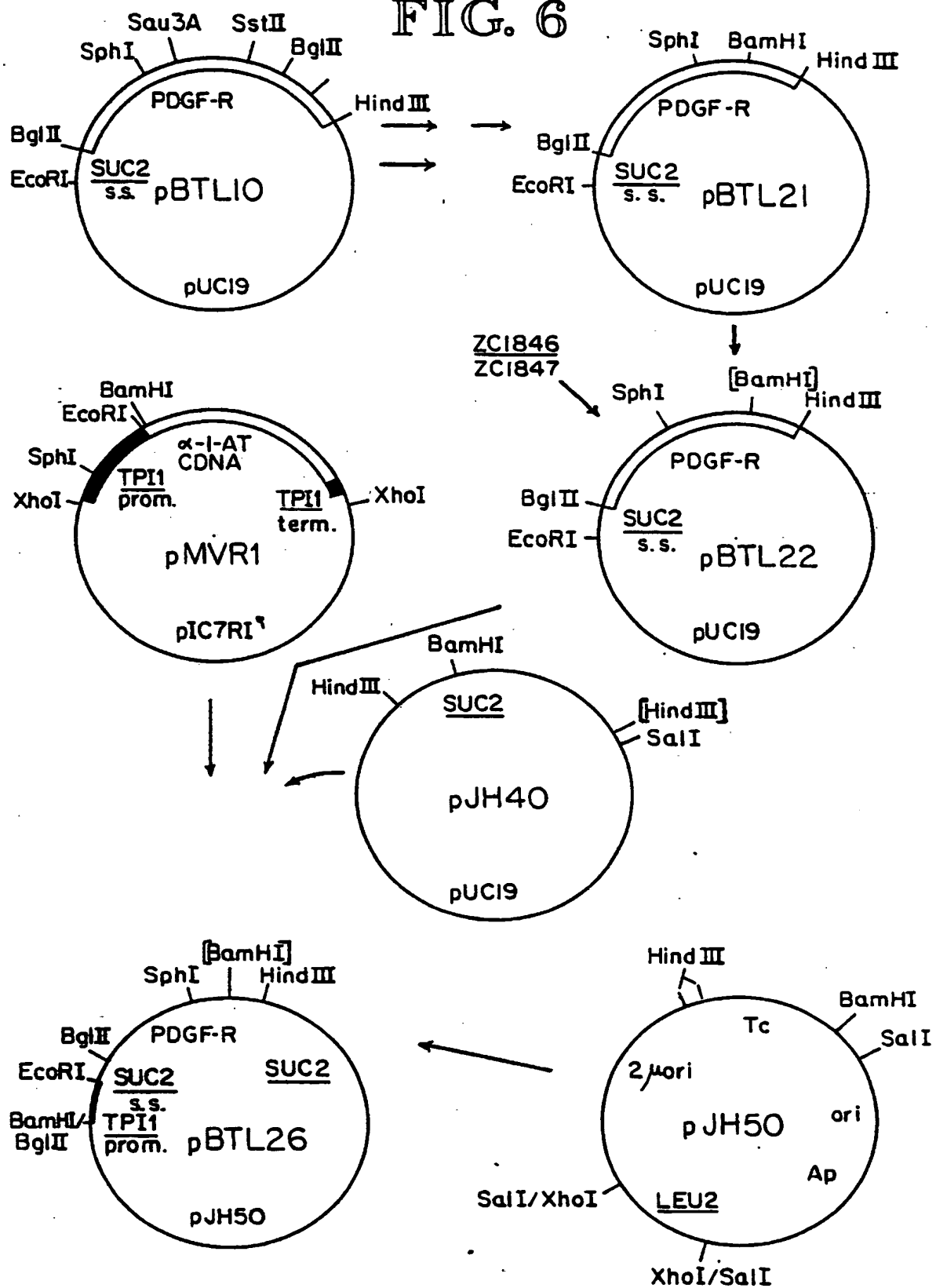


FIG. 7

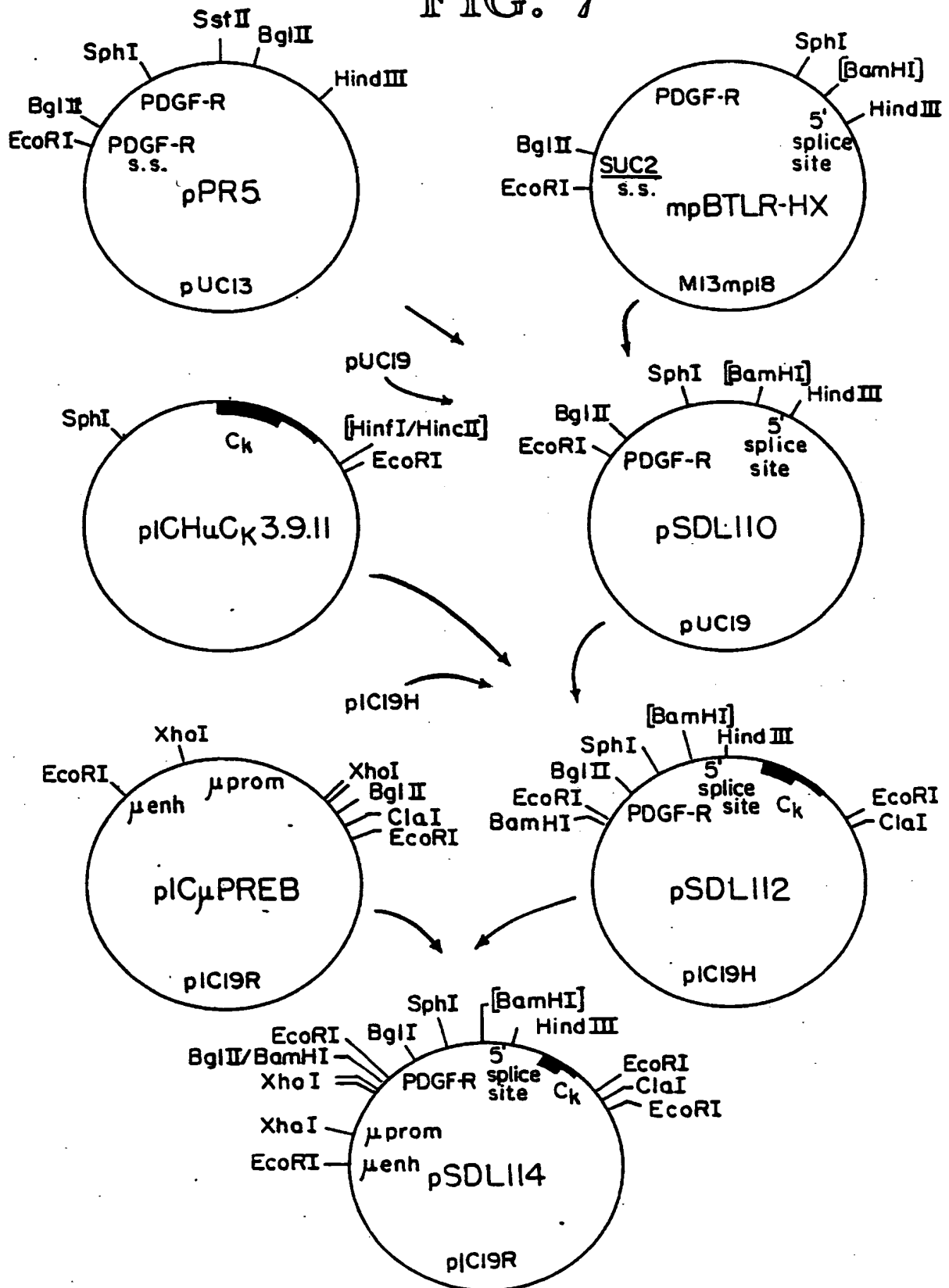


FIG. 8

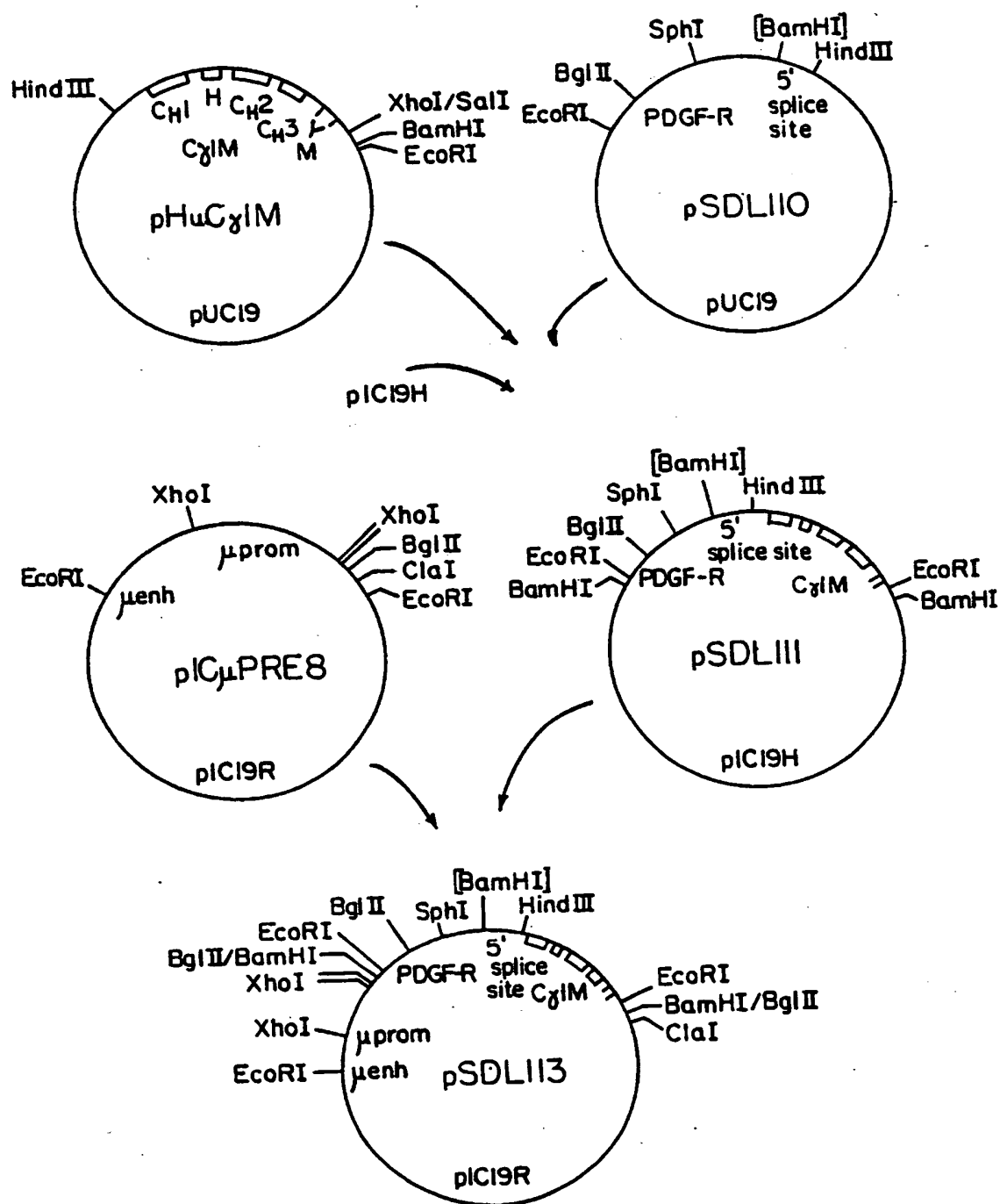
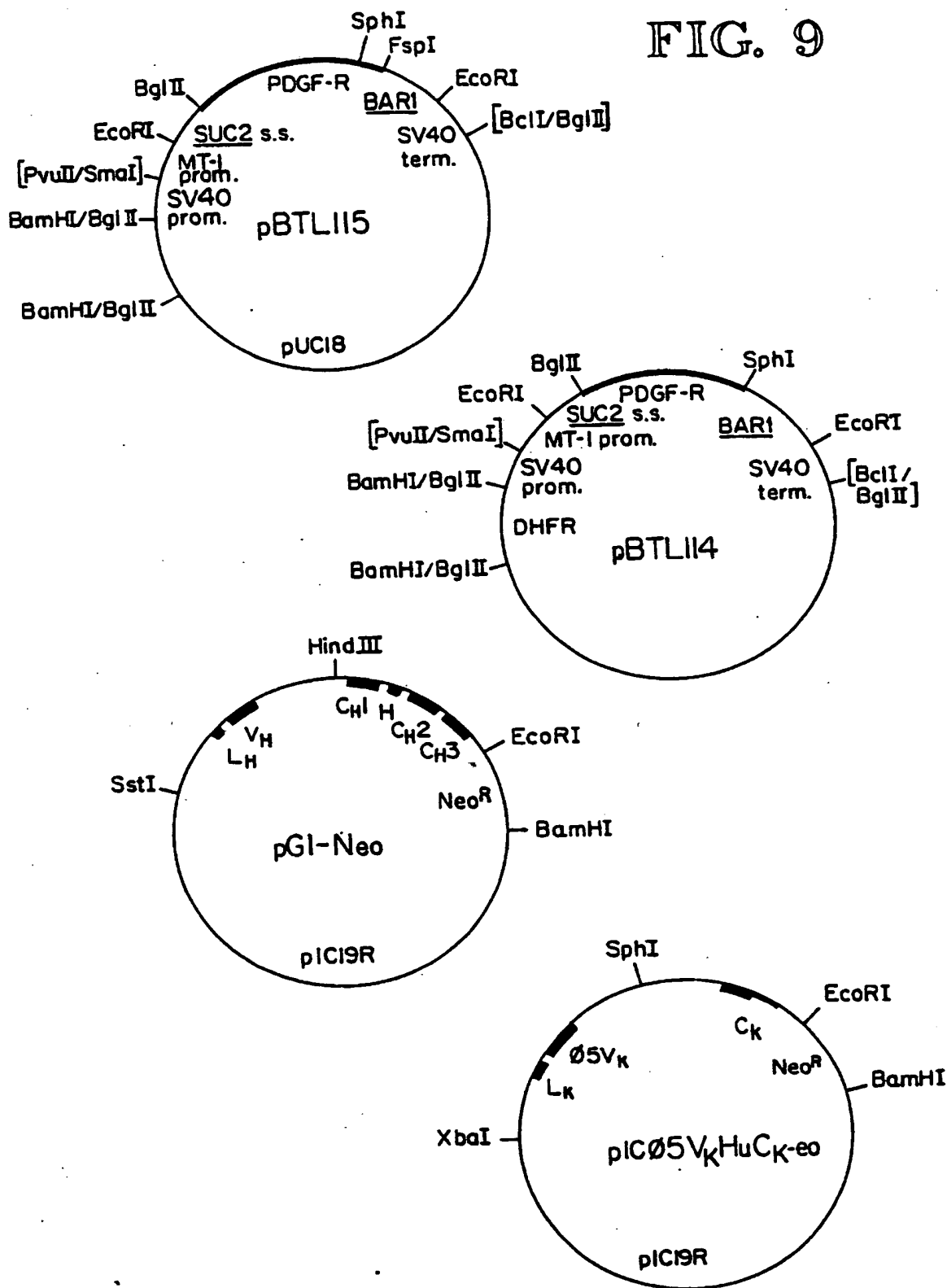


FIG. 9



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